EPIDEMIOLOGICAL AND LABORATORY STUDIES OF GROUP B BETA-HAEMOLYTIC STREPTOCOCCI

C.G. Cumming

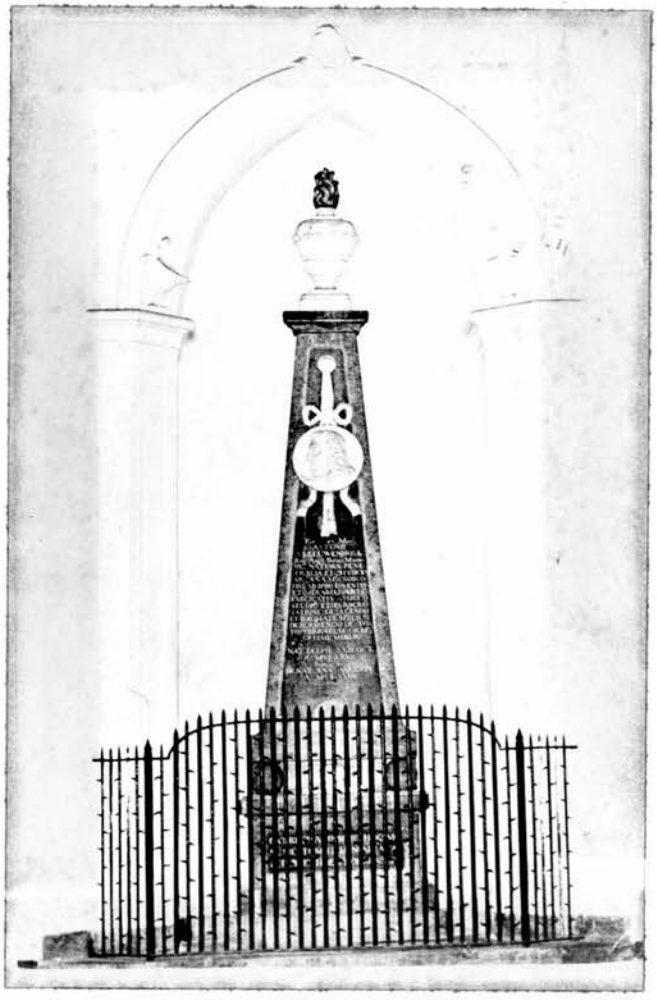
B.D.S.

Thesis presented for the degree of Doctor of Philosophy,

University of Edinburgh

1980

FRONTISPIECE



Monument to Antonie van Leeuwenhoek,

Delft, Holland, 1632-1723.

Acknowledgement

Dr Philip W. Ross has been a constant source of invaluable advice and encouragement throughout the course of these studies. To him I am greatly indebted. I am grateful to many members of the Bacteriology Department in the University Medical School, especially Professor J.G. Collee and Dr Ian R. Poxton for their continuous help and criticism.

Robert Brown in the Microbial Pathogenicity Research Laboratory has shown me the value of organisation in the laboratory and to him I am also grateful.

I am grateful to Hannah Lough and Aileen Nicoll for their extreme patience and assistance in the laboratory throughout the studies.

Professor J.C. Southam of the Department of Oral Medicine and Oral Pathology has kindly allowed me considerable freedom from the Department during the last five months and I am additionally grateful to him for advice in preparation of the thesis.

Margaret Lynn has given me unstinting support over many years in all respects of my studies and I am indebted to her.

Thanks are due to Ian Goddard and Robert Renton for illustrations, and to Vivien McGrath for a rapid type-script.

Throughout these studies the financial assistance of the Edinburgh Faculty of Medicine Scholarship Fund, and the Dean of Dentistry, has been greatly appreciated.

The results of some of the work presented in this thesis have already been reported. The references are:

(i) ROSS, P.W. & CUMMING, C.G. (1979). Recovery of group-B streptococci from swabs and transport media. In Pathogenic Streptococci, Reedbooks, p.180.

(ii) CUMMING, C.G. & ROSS, P.W. (1979). Evaluation of bacteriological swabs and transport media in the recovery of group B streptococci on laboratory media. Journal of Clinical Pathology 32, 1066-1069.

(iii) ROSS, P.W., NICOLL, A. & CUMMING, C.G. (1980). Use of the Streptosec test for grouping beta-haemolytic streptococci. Journal of Clinical Pathology 33, 691-693.

(iv) CUMMING, C.G., ROSS, P.W., POXTON, I.R. & McBRIDE, W.J. (1980). Grouping of beta-haemolytic streptococci by enzyme-linked immunosorbent assay. Journal of Medical Microbiology.

(v) CUMMING, C.G. & ROSS, P.W. (1980). Grouping of β-haemolytic streptococci by enzyme-linked immunosorbent assay (ELISA). International Association of Dental Research, British Division, Abstract no. 170 (Journal of Dental Research, in press).

(vi) CUMMING, C.G., ROSS, P.W. & POXTON, I.R. (1980). Immunochemical studies on the cell-wall antigen of group B streptococcus, type Ib. Journal of General Microbiology, in press.

(vii) ROSS, P.W. & CUMMING, C.G. (1980). Isolation of bacteria from the upper respiratory tract. Journal of Antonie van Leeuwenhoek, in press.

(viii) CUMMING, C.G., ROSS, P.W. & POXTON, I.R. (1980). Isolation of polysaccharide antigens from the cell wall of group B streptococcus, type Ib. Journal of Antonie van Leeuwenhoek, in press.

(ix) ROSS, P.W. & CUMMING, C.G. (1980). Optimal methods for the isolation of groups A, B, C and G streptococci. Journal of Otology and Laryngology, in press.

(x) CUMMING, C.G. & ROSS, P.W. (1980). The prevalence of group B streptococci in the upper respiratory tract of schoolchildren. Journal of Infection, submitted.

The investigations and procedures described in this thesis were designed and performed by the author. No portion of this thesis has been submitted in support of an application for another degree or qualification of this or any other university or institution of learning.

TABLE OF CONTENTS

Table of Contents (contd.)

LIST OF ILLUSTRATIONS

List of illustrations (contd.)

List of illustrations (contd.)

List of illustrations (contd.)

List of illustrations (contd.)

List of illustrations (contd.)

List of illustrations (contd.)

SUMMARY

The literature on the epidemiology, pathogenesis, and laboratory identification procedures for group B, beta-haemolytic streptococci (*Streptococcus agalactiae*) is reviewed.

Sampling methods for optimum isolation of beta-haemolytic streptococci, particularly group B, were investigated and the role of transport media in maintaining the survival of these bacteria on swabs was assessed. The results indicated that in general, none of the recognised transport media preparations offered any advantage over the use of plain cotton-wool swabs in preserving beta-haemolytic streptococci during storage for periods up to 48h. Storage temperature of swabs did however have a profound effect on survival of organisms.

The prevalence and significance of group B streptococci (GBS) in the upper respiratory tract of a group of Edinburgh schoolchildren was investigated. During the period of study a number of sampling and laboratory techniques for the isolation and identification of GBS were compared. The overall carriage rate of beta-haemolytic streptococci in the throats of the children sampled and the role of this area in the isolation of GBS is discussed.

A new commercially-available kit for the identification of beta-haemolytic streptococci was compared with the standard 'Lancefield' technique for grouping streptococci. In addition, a system of streptococcal grouping based on a modified enzyme-linked immunosorbent assay (ELISA) is presented. The value of this technique was assessed against the two previously mentioned systems.

A degree of difficulty in obtaining the required specificity in the ELISA studies prompted further investigations into the immuno-chemical character of the cell wall of strains of GBS. Unlike many previous studies, cell walls were collected and purified by sodium dodecyl sulphate (SDS) treatment and the secondary wall polymers were separated from the peptidoglycan component by a variety of procedures. Extracted antigens were visualised by reacting with specific antisera in a crossed immunoelectrophoresis system. Further purification of antigen complexes were achieved by chromatographic methods and chemical analysis was performed using paper and gas-liquid chromatography. The relevance of the specific antigen complexes isolated from GBS cells in relation to serological grouping and typing methods is discussed. Comment is made on the possibilities of further applications of these studies.

Chapter 1

Introduction

Introduction

Group B streptococci (Streptococcus agalactiae)

Streptococcus agalactiae was first described by Nocard and Mollereau in 1887 in association with bovine mastitis, and for many years these organisms were considered to be primarily animal pathogens with little predeliction for humans. In 1937, Colebrook and Purdie related group B streptococci with disease in man. However, those diseases that have attracted most attention because of their serious nature are septicaemia and meningitis in neonates.

Cultural and biochemical reactions

Group B streptococci grow rapidly on media enriched with blood, serum or glucose (Fig. 1.1). Colonies on blood agar are approximately 1 mm in diameter after aerobic incubation for 24h at 37°C, and are generally soft, low convex, grey-white and glossy in appearance. Haemolysis on blood agar is variable from none to clear beta-haemolysis. Brown (1920) was able to distinguish human strains from bovine strains of group B streptococci by virtue of the characteristic size and uniformity of the haemolytic patterns produced by organisms cultured from humans. The pathogenic strains of bovine origin showed considerable variety and irregularity of haemolytic areas when grown on blood agar.

In 1937, Brown described a double zone of haemolysis associated with human group B strains which appeared to be influenced

by several factors, such as the pH of the medium and the type of blood used. The majority of group B streptococci, regardless of their haemolysis on blood agar, produce a zone of enhanced haemolysis of sheep erythrocytes in the presence of staphylococcal beta-lysin, a phenomenon known as the CAMP reaction (see Chapter 3). Other specific biochemical aspects of group B streptococci are discussed in Chapter 3 and include their ability to hydrolyse sodium hippurate to glycine and benzoates; pigment production in growth media containing starch; and the ability to produce a lowering of pH in glucose broth. The fermentation reactions of these organisms of human origin were summarised by Parker and Ball (1976). They showed that most of the strains examined hydrolysed arginine, gave a negative Voges-Proskauer reaction, and fermented trehalose, salicin and sucrose, but not sorbital, mannitol, raffinose, inulin, arabinose or melizitose. Lactose was fermented by 13% of the cultures.

Serological differentiation

In 1924, Hitchcock demonstrated that most beta-haemolytic streptococci of human origin possessed a serologically active polysaccharide antigen. Lancefield (1933) showed that reaction of the extracted polysaccharide antigen with specific antisera raised against whole streptococcal cell preparations enabled the separation of streptococcal strains into separate serological groups. These streptococcal groups were subsequently known as the 'Lancefield groups'. Streptococcus agalactiae was designated as group B streptococci.

The serological typing system for group B streptococci was described later by Lancefield in 1934 and three serological types (I, II, III) were recognised. On the basis of type-specific protein and carbohydrate antigens serotype I has been sub-divided to include types Ia, Ib and Ic. The antigenic constitution of group B streptococci is discussed in detail in Chapter 4.

Spectrum of disease

During the last twenty years reports have shown that a wide spectrum of diseases in both adults and neonates can be attributed to group B streptococci (see Chapter 3). Infections in adults appear to be less frequent and tend to have a bimodal age distribution. Generally, those groups at most risk are young, previously healthy female populations in which group B infection occurs as a complication of pregnancy or the post-partum period, and an older age group where the host has an underlying deficiency or disease.

It is neonatal disease that has currently generated considerable attention. The introduction of antibiotics in the 1940's was associated with a dramatic reduction of incidence of neonatal infections due to Streptococcus pyogenes. In the late 1940's this pathogen was replaced by coliform organisms as the most common aetiological agent of neonatal meningitis and septicaemia. In the late 1950's and early 1960's, however, Staphylococcus aureus, in addition to coliforms, became recognised as the significant pathogens of neonates. During the past 10 years a considerable weight of evidence (see Chapter 3) has indicated that group B

streptococci are increasingly implicated in neonatal disease and are presently thought to cause the majority of cases of meningitis and septicaemia of the newborn. Baker et al. (1973) suggested that group B neonatal infections could be separated into distinct syndromes, an "early onset" or septicaemic form frequently occurring within the first 36h of birth, and a "late onset" or meningitic form affecting babies up to several months of age. The present position, however, recognises that no sharp demarcation between the two syndromes should be attempted; suffice to say that approximately 70% of cases occur within the first three days of life.

The general clinical picture of those babies affected in the first few days of life is that of undifferentiated sepsis with little or no inflammatory response on the part of the host. Apnoea is frequently present and symptoms are often indistinguishable from hyaline membrane disease. Older infants are usually able to localise the infection and inflammatory foci may be found within the meninges. The overall prognosis of the meningitic form of group B disease is significantly better than that of early onset septicaemia.

AIMS OF THE THESIS

The aims of the investigations presented in this thesis were:

(1) to investigate and define optimum sampling and identification techniques in the clinical isolation of beta-haemolytic streptococci, and in particular Lancefield's group B.

(2) to ascertain the prevalence of group B streptococci in the upper respiratory tract of a sample of schoolchildren, and

to assess the importance of the throat a s a source or reservoir of these organisms.

(3) to develop and compare improved techniques for serological identification of group B streptococci.

(4) to demonstrate the immunochemical profiles of certain antigenic carbohydrate complexes in the cell wall of various serotypes of group B streptococci.

Fig. 1.1 Colonial appearance of Streptococcus agalactiae (strain 090R) on 10% human blood agar.

Chapter 2

Evaluation of bacteriological swabs and transport media in the recovery of beta-haemolytic streptococci on laboratory media.

Introduction

The accurate clinical diagnosis of a microbial infection is dependant both upon a reliable method of sampling from the infected host, as well as efficient laboratory procedures which allow identification of the causative pathogenic agent in the specimen. Factors to be considered in the sampling technique include the time of culture, site of culture, and method of culture. Ferrieri et al. (1977) in their studies on pregnant women, reported that 20 women were found to be positive for group B streptococci (GBS) during labour but were GBS negative during the third trimester of pregnancy. Of the babies born, 19 who were GBS negative at birth had become positive by the time of discharge, whereas 14 babies originally positive became negative by the time of discharge. Similarly, the Commission on Acute Respiratory Diseases in the U.S.A. (1949) after taking repeated throat cultures from men in isolation concluded that a single throat culture was a poor index of the prevalence of beta-haemolytic streptococci and pneumococci in the upper respiratory tract, and that a true picture of the carrier state could only be obtained by repeated cultures over a period of a few weeks.

The site from which the sample is taken is also important. Anthony et al. (1975) stated that urethral cultures from females were more often positive for GBS than vaginal cultures, and a study by Ross (1971) of 20 schoolchildren who were known carriers of beta-haemolytic streptococci showed that not all areas of the pharynx were regularly colonised by these organisms.

The type of specimen taken from the patient and its transfer to the diagnostic laboratory for culture has an important bearing in obtaining a meaningful bacteriological result. The sample destined for culture is of no value if there is considerable loss of viability during transit to the laboratory. Generally, direct culture at the bedside or collection of clinical material such as pus, faeces or urine permits a reliable diagnosis to be made. The nature of many bacterial infections and epidemiological surveys however, precludes the collection of such clinical samples and in these cases the swab has been used with varying degrees of success.

The type of swab most commonly used consists of a small pledget of material, usually cotton wool wrapped smoothly and tightly around the roughened end of a wooden, wire or plastic applicator in such a way that no part of the end of the applicator is left exposed. This construction minimises the risk of the swab material being detached during the collection of the specimen, particularly important for nasopharyngeal, cervical and rectal swabs.

The earliest accounts describing the use of cotton wool swabs in clinical pathology were given by Councilman (1893) in the U.S.A. and by Preston (1896) and Hewlett and Nolan (1896) in England who recommended their use in the diagnosis of diphtheria. At this time other workers in Europe were using a variety of bizarre techniques for the collection of infected material. In France, Roux and Yersin (1890) described an apparatus consisting of platinum wire flattened at one end for sampling diphtheritic membranes.

Goldscheider (1893) in Germany used a platinum scoop and Hardman (1895) collected the discharges on white paper which was then dried in front of a fire and sent to the laboratory by post. Perhaps the most eccentric method (Hamilton, 1895) was the use of a goat's-hair brush to collect clinical specimens. By the early 1900's cotton wool swabs had gained wide acceptance amongst bacteriologists (Kolle and Wassermann, 1903), but little attention was directed towards the relative efficiency of these appliances. Van Reimsdijk (1924) studied the survival times of Corynebacterium diphtheriae on swabs and found that by inserting the swab into serum-agar gel the survival of these organisms was maintained for 24h at room temperature. Similarly, Downie (1940) found that Neisseria meningitidis could be cultured from nasopharyngeal swabs after 24h providing the swab was placed in contact with a blood-agar medium contained in the swab tube during the holding period. The increased viability and seemingly protective effect conferred on organisms by placing the swab in contact with serum and blood agar was partially explained by Pollock (1948) who demonstrated that the long-chain unsaturated fatty acids present to varying degrees in untreated cotton wool had a bacteriostatic effect upon Streptococcus pneumoniae and Mycobacterium tuberculosis. An important controlled study to examine the survival of a range of common pathogens on cotton wool swabs was reported by Rubbo and Benjamin (1951). Initial results indicated that the survival time of Gram positive bacteria including Streptococcus pyogenes up to 48h largely depended upon the batch of cotton wool used in swab construction, and on the presence of moisture which tended

to accelerate rate of death. Storage of swabs at 4°C also appeared to prolong survival times of Streptococcus pyogenes. In an attempt to counteract the obvious batch variation in cotton wool, sample swabs were dipped in ox serum and dried before use. Significantly increased survival times of all bacteria tested except Neisseria strains were obtained from the treated swabs in comparison with the plain cotton variety, and the former's superiority was confirmed in a subsequent clinical trial. The protective effect attributed to serum coated swabs was not obtained by neutralisation of toxic lipid or water-soluble substances present in cotton wool since ether and water extraction of the wool failed to alter survival when compared with untreated samples. The variable quality of cotton wool used for swabs was also noted by Mair and McSwiggan (1965) who suggested that manufacturers should be obliged to conform to a defined standard of swab production. White (1965) suggested however that the quality of cotton wool was less important than the method used for sterilisation of the swab. Comparison of commercially-produced untreated cotton swabs sterilised by different methods indicated that gamma-irradiated swabs had a pronounced antibacterial effect, whereas similar swabs sterilised by steam achieved an acceptable recovery of bacteria. These results were later confirmed by a number of tests performed by Dadd and colleagues (1970). Anderson (1965) attributed this antibacterial effect to the low pH of processed cotton wool rather than to the effect of the gamma-radiation.

The initial doubts expressed concerning the reliability of plain cotton wool swabs popularised the practice of treating cotton

wool to overcome any intrinsic inhibitory factors (Cruickshank, 1953). Stuart and colleagues (1954) obtained improved results by boiling cotton in phosphate buffer, pH 7.6, and then treating with charcoal which absorbed fatty acids, and Cooper (1957) in a study comparing the recovery of both common enteric and upper respiratory tract pathogens from plain cotton wool, serum coated, and charcoal impregnated swabs recommended that for streptococci in particular, charcoal swabs were advantageous. The explanation offered for this conclusion was that charcoal swabs tended to retain moisture for longer periods than other swabs, and that "loss of moisture is undoubtedly deleterious to streptococci". This finding directly contradicted the results presented earlier by Rubbo and Benjamin (1951) and is especially surprising when later work (to be discussed) is taken into account.

Further evidence relating to the efficiency of charcoal swabs in maintaining viability of streptococci was reported by Bartlett and Hughes (1969). In their experiments, survival of Streptococcus pyogenes on different swabs for periods up to 24h was studied. Plain cotton wool swabs proved to be completely unsatisfactory, failing to yield any growth on plating at 4h. Charcoal-coated wool gave an acceptable recovery rate up to 4h, after which there was a dramatic decline in numbers of organisms recovered. Serum-coated swabs although preserving viability for 24h permitted massive overgrowth by other organisms. A consistently high recovery of organisms for the duration of the tests was obtained from cotton wool swabs soaked in 20% bovine albumin (fraction V) and sterilised by steam. A similar study (Ross and Lough, 1978)

did not detect any difference in recovery of upper respiratory tract pathogens held for 48h on serum-coated or plain phosphate-buffered cotton wool swabs. The number of organisms recovered with 8h ranged for 4-9% of the initial inoculum taken up by the swab; after 48h swabs yielded between 1-5% of the original inoculum. Generally, storage of swabs at $4^{o}C$ produced more reliable recovery rates than those held at room temperature, but it was noted that the refrigerated group remained moist over the 48h period.

The question of whether organisms in transit to the laboratory should be kept in a dry or moist environment has received considerable attention, especially in the U.S.A.. Following the studies of Stark and Herrington (1931) illustrating that streptococci could be cultured after storage for 97 days under dry conditions, many workers have presented convincing evidence to show that streptococci on swabs held in silica gel crystals remained viable for considerably longer periods than those held in moist conditions. Hollinger and Lindberg (1958) and Hosty et al. (1964) were among the first to point out the advantages of this technique. Redys and colleagues (1968) suggested that in addition to increasing isolation rates and survival times of beta-haemolytic streptococci, throat swabs kept dry by exposure to silica gel prevented overgrowth of other commensal flora on the swab. By this method, a more accurate quantitative analysis of organisms present could be obtained.

In conditions more favourable to bacterial multiplication occurring on the swab, such as in tropical areas, the use of silica gel was shown to offer considerable advantages over swabs

transported in a moist state (Taplin and Lansdell, 1973). It is paradoxical however, to find that recent literature states that in an epidemiological survey of schoolchildren in New Zealand to ascertain carriage of group A streptococci in throats, the presence or absence of silica gel did not significantly influence the numbers of streptococci isolated (Martin et al., 1977).

An alternative approach towards the elimination of possible deleterious effects of humidity on clinical samples during transport to the laboratory was adopted by Hollinger and Lindberg (1958). Throat swabs were used to inoculate a sterile filter paper strip by streaking the swab heavily over the surface of the paper. This was air dried for 2-3 min, placed between aluminium foil, and sealed in an air-tight bag. Strips were held 2-17 days prior to culturing. Results showed no significant difference between numbers of group A streptococci isolated by the filter paper strip method and swabs plated immediately after sampling. Recovery of streptococci of groups B, C and G by the filter paper method in this study were inconclusive however and further studies were suggested. An extensive survey of 2,262 throat cultures (Smith et al., 1965) found that the filter paper method, although designed primarily for transport of specimens through the mail, was equally as suitable as a primary streak plate method when used under hospital conditions.

The filter paper strip technique has also been applied with mixed success to the collection and transportation of enteric pathogens in stools (Joe, 1950; 1956). The drying process did not appear to have any adverse effect on the recovery of Salmonella strains, unlike Shigella organisms which had limited survival on

dried paper (Bailey and Bynoe, 1953).

So far, this introduction has concentrated mainly on the merits and problems involved in the development of cotton wool as a suitable material for swabs. At the same time as these studies were progressing, other workers attempted to utilise different types of material for sampling procedures.

Initial experiments (Higgins and Marcella, 1950) to examine the possibilities for use of calcium alginate wool in swabs showed that it contained lauryl pyridinium bromide, a quaternary ammonium compound that is bactericidal. After thorough purification it was reported that the recovery of Staphylococcus aureus from calcium alginate swabs was almost double that experienced with cotton wool swabs (90.7% and 49.9% respectively). Tests with E. coli showed that the recovery rate dropped, but still remained in favour of calcium alginate (89.9% compared with 67.0% for the cotton wool).

Cain and Steele (1953) at a U.S. Naval base carried out a survey to assess the residual flora left on eating utensils after normal washing. Recovery of unspecified organisms was significantly greater from alginate swabs in comparison with the cotton wool variety.

A wide range of synthetic fibres including dacron, rayon, nylon, terylene, ulstron (polypropylene), tricel, and fortrel (polyester) have all been tried at some stage as swab materials. Dacron in particular, especially if held in silica gel during storage, has been recommended (Hollinger and Lindberg, 1958; Hosty et al., 1964). Fortrel swabs were clearly superior to those made with dacron in maintaining viability of group A streptococci

(Ellner and Ellner, 1966). The impressive study of Dadd et al. (1970) to investigate the survival of group A streptococci indicated that swabs made with synthetic fibres performed equally well in preserving high viability of the organisms. Treated cotton wool swabs were less efficient in this respect, whereas calcium alginate proved to be totally unsuitable for use as dry swabs. A slight increase in survival of streptococci from alginate swabs could be achieved by immersing the swab in a solution containing 0.5% (w/v) sodium hexametophosphate and 0.5% (w/v) sodium citrate which dissolved the fibres. The resultant suspension was then plated directly onto blood agar. Despite this modification an acceptable recovery of streptococci was not produced.

In further attempts to minimise the effect of environmental factors affecting survival of organisms in transit, a number of workers have suggested that swabs should be placed into an inert transport media before culturing.

The best known medium used for this purpose was described by Stuart (1946) in Glasgow. Media constituents were described in detail by Stuart et al. (1954). The value of this medium for maintenance of Neisseria gonorrhoeae during transport is well documented (Le Minor et al., 1949; Wilkinson, 1951; Alexander, 1952; and Stuart, 1956). During a carefully controlled laboratory trial, Cooper (1957) commented on the remarkable ability of the Stuart swab kit in preserving survival of common upper respiratory tract pathogens including beta-haemolytic streptococci. Swabs harbouring these organisms produced growth for periods as long as 12 weeks if placed in the medium. Taplin and Lansdell (1973)

abandoned the use of Stuart's transport medium in a field study of streptococcal carriage of personnel in Vietnam because of the poor recovery of organisms from clinical lesions, and over-growth by extraneous gram-negative bacteria and moulds during transportation. Similar findings were also reported by Amies and Douglas (1965). Amies (1967) later claimed that a modification of the formula for Stuart's medium prevented multiplication of organisms held on swabs in this medium but his results were inconclusive.

A specialist medium which was designed to select and maintain viability of streptococci in particular was developed by Pike (1945), but little mention of its efficiency as a suitable transport medium is found in current literature.

AIMS OF THIS STUDY

The aims of the present study were:

1) to compare the effectiveness of a number of commercially available swabs in the recovery of beta-haemolytic streptococci
2) to assess the protective effect offered by different types of transport media on viability of streptococci stored on swabs
3) to investigate different conditions for storage of swabs.

Materials and Methods

Bacteria

Five strains of group A, 5 strains of group C and 5 strains of group G beta-haemolytic streptococci, all isolated from the upper respiratory tract of patients attending the Royal Infirmary of Edinburgh (RIE) were studied. Twenty five strains of group B streptococci were collected from various sources as follows. Five reference strains representing the different serotypes (Ia, Ib, Ic, II, III) were supplied by Dr J. Stringer, Streptococcal Reference Laboratory, Colindale, London; 10 strains were isolated from the upper respiratory tract of patients attending the RIE, and a further 10 vaginal strains were isolated from pregnant women attending ante-natal clinics in the same hospital.

All strains were maintained on nutrient agar slopes in screw-capped vials and stored at $4^{o}C$.

Growth media

Todd-Hewitt broth (Oxoid Ltd, London) made up in 1 litre volumes and sterilised by autoclave at $121^{o}C$ for 20 min was routinely used for all liquid cultures. Blood agar was prepared by adding human blood to Columbia agar base (Oxoid Ltd, London) to a final concentration of 5% (v/v). Approximately 15ml of agar were dispensed into each plate; and plates were stored at $4^{o}C$.

Transport media

Amies' transport media (Amies, 1967) was obtained already sterilised by ethylene oxide in sealed packs from Exogen Ltd,

Glasgow, and Medical Wire and Equipment Co, Wiltshire. Pike's medium (Pike, 1945) was prepared in the laboratory and contained 5% (v/v) human blood agar supplemented with 1 in 1,000,000 crystal violet and 1 in 16,000 sodium azide. Stuart's medium (Stuart, 1946) was prepared using Stuart's transport medium modified (Oxoid CMIII) and was sterilised by autoclave.

Pike's and Stuart's media were dispensed into test-tubes in 5ml volumes and stored at $4^{o}C$ until use.

Silica gel crystals, coarse grade (B.D.H. Chemicals Ltd, Poole) were dispensed into test-tubes to a depth of 4cm and sterilised in a hot-air oven at $160^{o}C$ for 1h.

Swabs

Five types of commercially available swabs not held in transport media were tested. These swabs had been sterilised by ethylene oxide and were stored in sealed plastic containers until use.

Plain, buffered, absorbent cotton wool

Charcoal-coated absorbent cotton wool

Albumen-coated absorbent cotton wool

Calcium alginate

Dacron

Swab - transport media kits

These were as follows;

Buffered cotton wool swab in Amies' medium

Buffered cotton wool swab in charcoal Amies' medium

Calcium alginate swab in Amies' medium

Dacron swab in Amies' medium

Dacron swab in charcoal-Amies' medium

These kits are commercially available. The second group of swabs in media were prepared in the laboratory and comprised;

Buffered cotton wool swab with silica gel particles

Buffered cotton wool swab with Pike's medium

Buffered cotton wool swab with Stuart's medium

Saliva

Saliva was collected from two individuals and pasteurised by heating at 60°C for 30 min. Each batch collected was tested for sterility and stored at 4°C until use.

Method

Streptococcal strains were incubated aerobically in 10ml of Todd-Hewitt broth (THB) for 18h at 37°C. The bacterial suspension was then vortexed thoroughly to disrupt cells growing in chain forms. Initial testing procedures indicated that a dilution of 10^4 of the original broth culture in THB resulted in a countable number of organisms for use in this series of experiments. Swabs were loaded with an identical inocula consisting of one drop (0.02ml) of culture from the 10^4 broth dilution and four drops (0.08ml) of either saline (0.85% w/v NaCl) or pasteurised human saliva. The inoculum was carefully mixed on a small area of the inner surface of a sterile plastic petri-dish and then taken up by the swab. During the loading process the swab was rotated to ensure an equal distribution of organisms throughout the swab material. Pairs of swabs were either streaked out twice onto both halves of 5-10% human blood agar plates, or were placed into sterile plastic containers or transport media and held at room

temperature for a prescribed time before plating. The holding times were 8h, 24h and 48h.

To compare the effect of temperature on viability of the strains, duplicate sets of swabs with the same inoculum size were prepared and held for the same times at $4^{o}C$ before plating. During the plating procedure swabs were constantly rotated and the whole surface of the medium was covered. Plates were incubated aerobically overnight at $37^{o}C$ and surface colony counts were performed next morning.

The inoculum dilution was arranged so that swabs plated at 0h would yield counts of between 50-500 colonies. Fewer than 50 colonies was statistically insufficient and numbers in excess of 500 colonies could not be counted accurately. The initial number of organisms taken up by each swab was determined by surface viable counts from 10^{4} to 10^{5} dilutions of the original overnight culture. This was obtained by a spreadplate procedure using a sterile glass spreader.

Finally, at every time interval the average chain length of each colony-forming unit was calculated for the different swab types by gently rotating the swab head on a glass microscope slide and microscopic examination after Gram staining.

This study was carried out over a period of 10 months. For statistical reasons, a minimum of 15,000 swabs were streaked onto 30,000 half blood agar plates during the course of the investigation.

Statistical analysis

To evaluate the effects of time, temperature, type of swab

and inoculum size on the recovery rate of each group of organisms, data were combined and analysed as a randomised block experiment, the treatments being time, temperature and plating. Experimental blocks were the various groups of organisms. 2 X Standard Error (SE) = significant difference.

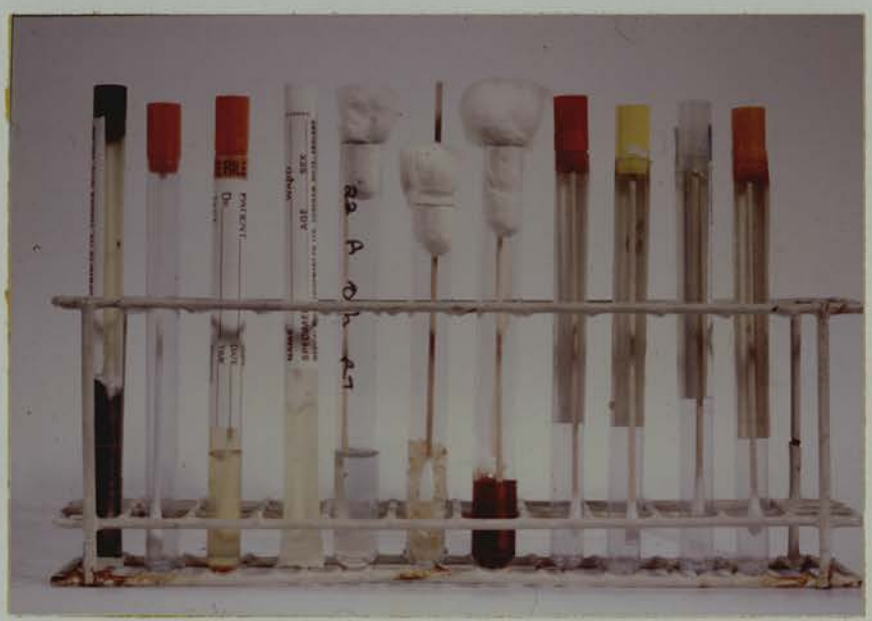


Fig. 2.1 Swabs with and without transport media, (from left to right) cotton wool in charcoal Amies'; plain dacron; cotton wool in Amies'; dacron in Amies'; cotton wool in Stuart's; cotton wool in silica gel; cotton wool in Pike's; plain cotton wool; albumen-coated wool; charcoal-coated wool; and calcium alginate.

Results

Swabs not held in transport media

The mean percentage recoveries of streptococci of groups A, B, C and G streptococci from swabs plated immediately after loading with the bacterial suspensions are shown in Tables 2.1 and 2.2. Each swab was plated out a second time onto the other half of a blood agar plate, and the mean percentage recoveries are indicated by values in parentheses.

It can be seen that little difference in the recovery of streptococci from the 5 types of swabs on immediate plating was found, regardless of whether saline or saliva was used as the carriage fluid. The striking feature however, was that even if swabs were plated immediately after loading the sample, the recovery of organisms was only around 10% of the initial inoculum, 90% of bacteria were lost. Yield from second plating of swabs was also consistent, at approximately 50-75% of the recovery produced by the initial plating procedure. Calcium alginate swabs were tested with group B streptococci only following the manufacturer's withdrawal of the product from the market.

Tables 2.3 and 2.4 present results showing recovery of group A streptococci in saline and saliva from swabs held in their plastic containers for 8, 24 and 48h prior to plating. The effect of holding swabs at room temperature and $4^{o}C$ was also compared.

The four varieties of swabs tested not only maintained viability of bacteria up to 48h but in some cases allowed multiplication of organisms on the swab to occur. In Table 2.3, multiplication on albumen swabs stored at room temperature was

evident after 8h and plating at 24 and 48h produced confluent growth on the surface of blood agar plates. A similar range of recovery was obtained from dacron swabs at 24 and 48h.

A negligible effect by loading swabs with group A streptococci in saliva (Table 2.4) instead of saline was observed, with the exception of the charcoal coated swab which hardly maintained viability of organisms in saline at 48h. In the presence of saliva however, bacteria underwent considerable multiplication by 24h at room temperature.

The effect of temperature was clearly significant in the recovery of group A streptococci from swabs. In comparison with the storage of swabs at room temperature, recovery from swabs held at $4^{o}C$ was relatively constant throughout the 48h storage period, and no bacterial multiplication was observed.

Tables 2.5 and 2.6 show the yields of group B streptococci (GBS) from swabs held in plastic containers for times up to 48h. The level of recovery of cells loaded onto the swab in saline (Table 2.5) was maintained at about 10% of the initial inoculum for plain, albumen and charcoal swabs regardless of temperature. Calcium alginate swabs proved to be the least efficient and at 48h only 2-3% of the original bacterial number could be cultured. The dacron swab behaved unusually in that bacterial multiplication occurred after 8h on swabs stored at both room temperature and $4^{o}C$.

The presence of pasteurised saliva on albumen, charcoal and dacron swabs at room temperature enabled rapid multiplication of GBS during the first few hours of storage, resulting in confluent growth on blood agar after 8h. Calcium alginate swabs again

produced poor yields while the plain cotton wool variety maintained a consistent recovery of 10%. Similarly, yields from all swabs stored at 4°C were not significantly altered throughout the 48h period.

Initial recovery of group C streptococci at 0h and throughout the 48h holding period was found to be slightly greater than recovery of groups A and B streptococci held under similar conditions on identical swabs. Table 2.7 shows the yield of organisms loaded onto swabs in saline. Culture of plain swabs stored at room temperature for 48h produced confluent growth on blood agar indicating multiplication of organisms on the swab. Although recovery from plain swabs held at 4°C declined at each of the culture times, 10% of the initial sample was recovered on blood agar plates from the 48h swabs. Albumen swabs stored at room temperature again allowed massive multiplication of organisms throughout the 48h storage period. Generally, charcoal swabs stored at 4°C and at room temperature produced a consistently high recovery rate of organisms (13-16%) up to 48h, and likewise dacron swabs at 4°C gave essentially the same result. Dacron swabs held at room temperature however were unsuitable for use in prolonging viability of group C streptococci (2.1% recovery at 48h).

The recovery rate of group C organisms in saliva (Table 2.8) did not differ significantly from that of group C organisms in saline, with two exceptions. Both charcoal and dacron swabs held at room temperature produced confluent growth during the 8-48h period. The 4°C recovery figures for dacron (26% at 48h) also contrasted sharply with the same swabs loaded with organisms

suspended in saline, held under similar conditions.

The results presented for recovery of group G streptococci in saline and saliva (Tables 2.9 and 2.10) show a decrease in the overall recovery rate, in relation to results for groups A, B and C. Albumen swabs inoculated with streptococci in saline and held at room temperature allowed a degree of multiplication to occur on the swab, as was the case with dacron swabs loaded with bacteria in saliva and held also at room temperature. On no occasion, however, was multiplication sufficient to produce confluent growth on blood agar when the swab was cultured. Charcoal swabs proved to be totally unacceptable in producing a satisfactory level of recovery of group G streptococci.

Bacterial chain lengths

Following thorough mixing of the streptococcal broth suspension prior to inoculation of samples onto swabs, broth was examined microscopically and the streptococci were mainly observed singly or in pairs. At every time interval and for each type of swab not held in transport media, cells remained in single or pairs configurations.

Swabs held in transport media

Tables 2.11 and 2.12 illustrate the percentage recovery of group A streptococci held on cotton wool and dacron swabs in a variety of transport media for periods of up to 48h. The overall impression given by these results indicated that in general the use of transport media did not help to maintain viability of these organisms.

Immersion of cotton wool swabs in Amies' medium and storage for 24h at room temperature resulted in the loss of over 99% of organisms on plating which is clearly unacceptable. Recovery of saline-loaded streptococci from cotton wool swabs in charcoal Amies' media remained constant at 4-6% during 48h storage, contrasting with the yield obtained from swabs loaded with bacteria in saliva.

The presence of saliva or saline on dacron swabs held in charcoal Amies' media had a marked effect on recovery. Initial multiplication of cells in saliva was observed from 8h swabs followed by a gradual decrease in yield during the next 40h. Cells in saline were recovered in low numbers throughout the experimental period.

Pike's and Stuart's media enabled higher recoveries to be recorded but Stuart's medium allowed slight multiplication of bacteria to occur on the swab when stored at room temperature.

The surprising feature of these results was the almost total loss of group A streptococci from swabs held in silica gel crystals.

In Table 2.13, the percentage recovery of group B streptococci (GBS) loaded onto swabs in saline and held in transport media is presented. The results bear a close similarity to the recovery of group A streptococci from swabs in transport media. Low yields (1-5%) of GBS were obtained from cotton wool swabs maintained in both Amies' and charcoal Amies' media, whereas dacron swabs in charcoal Amies' had considerable advantages over the cotton wool variety in recovery of organisms. Swabs cultured

at 24 and 48h from Pike's and Stuart's media at room temperature all produced confluent growth on blood agar plated, indicating a marked degree of cellular multiplication on the swab.

Storage of swabs in silica gel crystals produced a very low recovery of GBS.

Tables 2.14 and 2.15 show the recovery of group C streptococci from swabs in transport media. Overall, higher recovery rates were obtained from swabs containing streptococci in saliva compared to the saline inoculated group. The effect of temperature during storage was also more evident especially in Table 2.15. Multiplication of cells occurred when organisms in saliva were held on dacron swabs in Amies' and on cotton swabs in Pike's and Stuart's media at room temperature. The same swab/media combination held at $4^{o}C$ yielded significantly lower numbers of bacteria. Similar patterns of recovery of group C organisms were obtained from the other transport media swabs in comparison with results previously described. It was interesting to note that swabs in silica gel performed very badly.

Finally, the percentage recoveries of group G streptococci from swabs in transport media are presented in Tables 2.16 and 2.17. Recovery rates of group G strains tested did not differ markedly from results already presented for groups A, B and C streptococci. Cotton swabs loaded with saliva and stored at room temperature in Pike's and Stuart's media again produced multiplication on culture, and silica gel had a deleterious effect on storage of group G streptococci on swabs.

Bacterial chain lengths from swabs in transport media

Samples from each type of swab in transport media were examined microscopically during the experimental period.

Bacterial cells from cotton wool and dacron swabs held in Amies' medium were observed mainly in chains of 4 cells after 24h storage. Cells from cotton wool swabs held in Pike's and Stuart's media were found in chains of up to 15 cells at 24h and 25 cells or more at 48h. A few single cells could be observed from cotton wool swabs held in silica gel.

Tables 2.1 to 2.17

These tables present data indicating the degree of recovery of beta-haemolytic streptococci (Lancefield's groups A, B, C and G) from swabs maintained under different conditions for times up to 48h. The following abbreviations are used:

RT = room temperature

ND = no data available

TNC = too numerous to count

(%) = percentage recovery from swabs on second plating

SE = standard error

Table 2.1 Mean percentage recovery of streptococci in saline from swabs plated at 0 hour.

	Streptococci			
	Group A	Group B	Group C	Group G
Plain	10.1 (5.7)†	9.9 (6.6)	13.5 (9.2)	9.1 (6.1)
Albumen	7.6 (4.3)	8.4 (6.4)	12.5 (9.7)	8.9 (5.3)
Alginate	ND ND	7.4 (4.6)	ND ND	ND ND
Charcoal	7.5 (4.0)	10.4 (6.4)	13.7 (9.2)	7.1 (4.7)
Dacron	6.4 (3.5)	10.8 (6.8)	12.7 (7.5)	7.2 (4.6)

Standard error = 1.0

† second plating values in parentheses

Table 2.2 Mean percentage recovery of streptococci in saliva from swabs plated at 0 hour.

	Streptococci			
	Group A	Group B	Group C	Group G
Plain	8.1 (5.5)†	9.3 (6.9)	13.7 (11.0)	8.0 (5.8)
Albumen	6.5 (4.3)	10.8 (7.2)	16.7 (12.1)	7.6 (5.1)
Alginate	ND ND	5.7 (3.1)	ND ND	ND ND
Charcoal	5.8 (3.9)	8.2 (5.8)	20.1 (11.2)	6.8 (4.4)
Dacron	3.4 (2.9)	6.3 (4.5)	8.6 (5.4)	4.0 (2.6)

Standard error = 1.0

† second plating values in parentheses

Table 2.3 Effect of time and temperature on percentage recovery of group A streptococci in saline from swabs not held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Plain	8.2	8.6	11.8	6.8	3.2	4.2
Albumen	24.4	5.1	TNC	9.0	TNC	6.2
Charcoal	7.5	8.1	7.3	9.7	1.0	4.2
Dacron	4.9	5.0	TNC	2.7	TNC	4.7

Standard error = 1.2

Table 2.4 Effect of time and temperature on percentage recovery of group A streptococci in saliva from swabs not held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Plain	10.4	7.1	23.4	8.6	3.0	7.3
Albumen	1.7	5.4	TNC	5.4	TNC	7.0
Charcoal	10.4	5.6	TNC	7.1	48.2	3.7
Dacron	13.8	3.5	TNC	4.4	TNC	4.4

Standard error = 1.2

Table 2.5 Effect of time and temperature on percentage recovery of group B streptococci in saline from swabs not held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Plain	12.9	8.0	9.4	9.5	7.6	9.7
Albumen	8.6	8.2	9.9	6.3	9.5	6.2
Alginate	5.3	5.7	4.9	3.5	2.3	2.6
Charcoal	9.7	10.7	11.0	9.3	9.2	7.6
Dacron	21.8	14.0	TNC	24.8	TNC	20.3

Standard error = 1.3

Table 2.6 Effect of time and temperature on percentage recovery of group B streptococci in saliva from swabs not held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Plain	10.2	10.6	9.8	8.7	11.9	8.4
Albumen	TNC	9.7	TNC	8.5	TNC	9.9
Alginate	5.9	5.4	6.2	4.3	3.4	3.7
Charcoal	25.1	7.9	28.4	7.3	TNC	5.5
Dacron	TNC	7.7	TNC	6.6	TNC	6.5

Standard error = 1.3

Table 2.7 Effect of time and temperature on percentage recovery of group C streptococci in saline from swabs not held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Plain	29.4	19.5	20.5	16.9	TNC	10.8
Albumen	TNC	14.7	TNC	28.8	TNC	30.0
Charcoal	15.7	16.6	5.9	14.0	16.1	12.9
Dacron	5.6	18.8	6.7	11.5	2.1	13.9

Standard error = 1.0

Table 2.8 Effect of time and temperature on percentage recovery of group C streptococci in saliva from swabs not held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Plain	23.9	9.3	TNC	22.0	TNC	9.5
Albumen	TNC	13.0	TNC	31.0	TNC	20.7
Charcoal	TNC	10.8	TNC	21.3	TNC	19.9
Dacron	TNC	9.5	TNC	24.1	TNC	26.0

Standard error = 1.0

Table 2.9 Effect of time and temperature on percentage recovery of group G streptococci in saline from swabs not held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Plain	9.5	6.8	4.3	5.0	4.1	5.1
Albumen	12.2	7.2	24.9	8.2	19.1	8.1
Charcoal	1.8	6.2	0.1	5.7	0.3	1.2
Dacron	6.1	5.7	7.3	10.7	4.5	8.8

Standard error = 1.0

Table 2.10 Effect of time and temperature on percentage recovery of group G streptococci in saliva from swabs not held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Plain	8.1	4.8	4.7	5.5	5.3	4.7
Albumen	12.4	7.2	13.8	7.2	15.3	6.9
Charcoal	1.6	5.3	1.3	4.2	-	2.5
Dacron	9.1	5.5	22.7	5.3	20.5	4.0

Standard error = 1.0

Table 2.11 Effect of time and temperature on percentage recovery of group A streptococci in saline from swabs held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Cotton-wool in Amies'	2.5	3.4	0.7	1.7	0.1	1.9
Cotton-wool in charcoal Amies'	3.3	4.0	1.3	3.6	2.1	1.0
Dacron in charcoal Amies'	6.3	3.7	5.0	3.6	4.9	4.1
Cotton-wool in Pike's	7.2	1.4	4.1	2.4	8.0	2.9
Cotton-wool in Stuart's	9.1	3.9	13.4	2.6	28.8	3.7
Cotton-wool in silica gel	All less than 1%					

Standard error = 1.4

Table 2.12 Effect of time and temperature on percentage recovery of group A streptococci in saliva from swabs held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Cotton-wool in Amies'	6.0	6.4	5.9	1.5	1.0	2.4
Cotton-wool in charcoal Amies'	7.9	7.5	3.5	5.0	13.8	8.1
Dacron in charcoal Amies'	53.2	11.5	21.3	8.3	13.4	8.8
Cotton-wool in Pike's	4.0	3.1	1.0	4.2	1.1	3.1
Cotton-wool in Stuart's	10.3	4.6	23.6	4.8	26.0	3.3
Cotton-wool in silica gel	All less than 1%					

Standard error = 1.4

Table 2.13 Effect of time and temperature on percentage recovery of group B streptococci in saline from swabs held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Cotton-wool in Amies'	3.3	5.0	2.6	3.5	1.4	2.8
Cotton-wool in charcoal Amies'	3.3	4.6	1.7	3.5	0.4	2.7
Dacron in charcoal Amies'	8.3	9.0	6.9	5.6	6.9	7.0
Cotton-wool in Pike's	3.4	3.6	TNC	3.5	TNC	3.4
Cotton-wool in Stuart's	4.4	3.0	TNC	2.8	TNC	3.0
Cotton-wool in silica gel	0.4	1.4	1.3	1.4	0.8	0.9

Table 2.14 Effect of time and temperature on percentage recovery of group C streptococci in saline from swabs held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Cotton-wool in Amies'	2.2	5.9	2.0	3.1	2.3	4.2
Cotton-wool in charcoal Amies'	2.9	3.5	4.2	1.8	18.1	1.3
Dacron in charcoal Amies'	6.4	4.4	5.1	2.3	3.4	2.4
Cotton-wool in Pike's	10.2	1.6	10.4	1.6	47.6	1.8
Cotton-wool in Stuart's	4.8	5.3	1.7	4.0	0.3	4.8
Cotton-wool in silica gel	0	0.6	0.3	1.5	0.2	0.9

Standard error = 1.2

Table 2.15 Effect of time and temperature on percentage recovery of group C streptococci in saliva from swabs held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Cotton-wool in Amies'	7.7	8.8	5.6	5.8	3.9	4.7
Cotton-wool in charcoal Amies'	5.8	4.1	6.7	2.8	1.4	2.5
Dacron in charcoal Amies'	11.6	5.7	40.0	4.0	16.2	4.6
Cotton-wool in Pike's	16.3	1.2	18.6	1.9	15.8	2.5
Cotton-wool in Stuart's	3.6	4.5	10.5	6.7	19.7	7.3
Cotton-wool in silica gel	All less than 1%					

Standard error = 1.2

Table 2.16 Effect of time and temperature on percentage recovery of group G streptococci in saline from swabs held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Cotton-wool in Amies'	1.5	2.5	2.7	1.0	4.0	1.9
Cotton-wool in charcoal Amies'	2.0	4.2	1.9	2.3	3.2	4.4
Dacron in charcoal Amies'	3.2	3.5	7.3	5.0	6.3	4.7
Cotton-wool in Pike's	7.3	4.6	14.2	3.3	11.9	4.1
Cotton-wool in Stuart's	3.3	3.7	5.2	2.2	14.4	2.3
Cotton-wool in silica gel	0.7	1.3	2.5	2.1	1.4	1.7

Standard error = 1.5

Table 2.17 Effect of time and temperature on percentage recovery of group G streptococci in saliva from swabs held in transport media.

	8 hours		24 hours		48 hours	
	RT	4°C	RT	4°C	RT	4°C
Cotton-wool in Amies'	3.9	3.6	5.3	3.6	8.2	3.6
Cotton-wool in charcoal Amies'	7.8	5.5	7.7	3.6	3.0	10.1
Dacron in charcoal Amies'	21.9	6.4	18.4	6.8	7.2	8.5
Cotton-wool in Pike's	4.8	4.4	14.0	8.6	15.1	4.7
Cotton-wool in Stuart's	6.2	5.2	21.1	3.1	20.8	4.0
Cotton-wool in silica gel	0.4	0.8	1.4	1.3	0.4	0.8

Standard error = 1.5

Discussion

The traditional means of obtaining information relating to the epidemiology of particular bacterial infections has relied to a large extent upon sampling amongst various population groups. Although bacteriological sampling techniques in certain types of surveys have varied, and indeed improved in recent times, the epidemiologist still depends to a large extent on the use of the swab as a method of collection and transportation of the sample to the laboratory. In view of the reported limitations of different types of swabs in maintaining viability of bacteria, it is reasonable to suggest that early surveys may have given a better indication of the efficiency of the sampling technique rather than meaningful information relating to the status of bacterial carriage in populations.

Factors described in the introduction to this chapter which may adversely affect recovery of organisms from cotton wool swabs include the batch of cotton wool used for the swab, presence of inhibitory fatty acid within the wool, sterilisation procedures, presence or absence of moisture on the swab, and storage temperature of the swab. Treatment of the wool by dipping in phosphate buffer at pH 7.6 or coating with albumen, serum and charcoal appeared to improve the performance of the swab considerably. Other workers suggested the use of synthetic fibres, and dacron swabs in particular have received considerable attention. Recent reports advocated the practice of storing swabs in transport media to eliminate possible deleterious effects of the environment on the sample. This technique has undoubtedly helped in prolonging

survival of some of the more fastidious organisms such as Neisseria gonorrhoeae strains but with regard to the isolation of the upper respiratory tract pathogens the use of transport media allows overgrowth of commensal bacteria on the swab.

The bacteriologist has therefore a wide range of sampling devices to choose from, not all of which have received detailed testing. The aim of this survey was to assess the suitability of the many swab and transport media options available and to ascertain the most efficient sampling appliances for recovery of beta-haemolytic streptococci, particularly group B (GBS). This was necessary in view of a projected survey of GBS carriage in schoolchildren (see Chapter 3).

The prominent feature of the survey was the great loss of organisms on initial plating of swabs. In the case of swabs not held in transport media this loss was approximately 90% of the initial inoculum after immediate plating, and up to 99% loss by 48h. Recovery from swabs at the second time of plating remained constant at 50-75% of the first plating value. On certain occasions all types of swab, especially those carrying streptococci in saliva at room temperature allowed bacterial multiplication to occur on the swab. This rarely occurred when swabs were stored at $4^{o}C$. From a clinical point of view the danger of a procedure that allows multiplication in the sample during transit is that other commensal organisms will also multiply and may obliterate or outgrow the causative pathogens, particularly if these are present in small numbers in the lesion.

With the exception of calcium alginate swabs which performed very poorly in the recovery of group B streptococci, the overall

conclusion is that recovery from plain, buffered absorbent cotton wool swabs was more consistent than that from charcoal swabs, and allowed less bacterial multiplication than dacron and albumen swabs. Bacteria in saliva were recovered in slightly higher numbers than those suspended in saline but saliva swabs were more prone to allow overgrowth of organisms. Storage of swabs at $4^{o}C$ was shown to be essential to obtain consistency of results and to prevent multiplication of cells on the swab.

An astonishing loss of streptococci held on swabs in transport media was recorded in this survey. Storage of cotton swabs in silica gel ensured an almost total loss of organisms on culturing even within the first 8h of the holding period, regardless of whether the swab was moistened or in a dry state immediately prior to plating. Recovery from cotton and dacron swabs in Amies' medium was significantly lower than from the same swabs not stored in transport media, and although Pike's and Stuart's media mostly allowed reasonable recovery, results were inconsistent and multiplication was very evident throughout the experiments.

On the basis of results presented it is suggested that transport media are of no benefit in maintaining the viability of beta-haemolytic streptococci in transit to the laboratory, and indeed their use at times may be detrimental to adequate recovery of organisms from swabs.

Collee et al. (1974) and Ross (1977) acknowledging that the swab is an inefficient sampling device for obtaining a primary culture plate indicated that at least a proportion of the losses in transit

that might be attributed to death of delicate organisms is really attributable to non-release of organisms onto plates (i.e. retention on the swab). For this reason, the practice of seeding the swab into broth after initial plating is well based, though this procedure does not allow direct bacteriological diagnosis and may lead to the complications that attend the selection of likely pathogens from mixed cultures.

Chapter 3

Upper respiratory tract carriage of group B streptococci

Introduction

1) Clinical significance of group B streptococcal diseases

Beta-haemolytic streptococci of Lancefield group B (GBS) have been implicated in human disease almost from the time when the precipitin-grouping technique (Lancefield, 1933) was first described. Since then additional reports have established that these organisms may occasionally cause severe infection in parturient women and neonates, and less frequently in other adult patients (Fry, 1938; Brown, 1939; Hill and Butler, 1940). In a review of newborn sepsis observed from 1933 to 1957 Nyhan and Fousek (1958) described six infants with sepsis or meningitis due to GBS in a group of 106 babies. Although the most frequent causative organism of neonatal sepsis was *E. coli*, this study was the first fully to implicate GBS as an important pathogen in neonates. A few years later Hood *et al*. (1961) and Keitel *et al*. (1962) confirmed the significance of GBS as a cause of infection amongst neonates. In 1964, Eickhoff and colleagues reported their experience with GBS infection in patients residing in Boston, U.S.A.. A total of 149 strains of GBS were isolated from 103 patients during life and from five patients at post-mortem examinations. More than half of the isolates were related to the female genital tract of parturient women, and their newborn offspring, while the remaining GBS isolates were collected mainly from sputum, surgical wounds, abscesses and urine. The range of infections present in these patients in whom GBS were considered to be the main aetiological agent included seven infants with neonatal sepsis, 10 women with septic abortions (four of whom were bacteraemic); five women with

chorioamnionitis that followed premature rupture of the membranes (one of whom was bacteraemic); eight diabetic adults with peripheral vascular disease; three women with pyelonephritis (one of whom was bacteraemic); and an elderly man with suppurative arthritis. Four of the infants with neonatal sepsis died, but all the other patients recovered. During the study period, Eickhoff et al. recorded that GBS were the most frequent single cause of neonatal infection and accounted for 5.2 cases of neonatal sepsis per 1,000 live births.

Maher and Irwin (1966) reported a single case of meningitis in a 42-h old infant, and Winterbauer et al. (1966) similarly reported two cases of neonatal meningitis due to GBS. An extensive survey conducted over six years by Butter and de Moore (1967) described 20 cases of meningitis of the newborn and four cases of neonatal sepsis attributable to GBS. A further 20 cases of bacteraemia and three of meningitis mainly in patients over 60 years old suffering from either diabetes or neoplasia were reported.

In the U.K., Jones and Howells (1968) reported two cases of neonatal meningitis, Rogers (1970) recorded 14 cases of infections due to GBS in newborn infants, and Harper (1971) described the plight of one infant who suffered from GBS meningitis.

By this time, it had become evident that essentially three distinct population groups were at most risk of infection by GBS.

(1) Infants up to the age of three to four months, especially those who had undergone a prolonged or abnormal birth.

(2) Pregnant and parturient women where GBS may be implicated in puerperal infection.

(3) An older age group where a predisposing condition, especially malignancy or diabetes, is often present, i.e. the "compromised host".

Considering the older age group first, Jelinkova et al. (1970) has amply described the types of infection directly attributable to GBS amongst this group of patients. In their study, the frequency of GBS in the total routine clinical material from hospitalised patients in Prague, Czechoslovakia, was approximately 0.5% over a five year period. Sites of pathological lesions brought about by GBS ranged from the respiratory tract (bronchopneumonia, tonsillitis and pharyngitis), urinary tract, myocardium, genital tract, meninges, ear, sinuses and conjunctivae.

In the early 1970's it was generally recognised that GBS caused serious neonatal disease of two types;

(1) Septicaemia, often with symptoms of shock or respiratory distress; and

(2) Meningitis.

Later however, American workers (Quirante and Cassady, 1972; Franciosi et al., 1973; Baker and Barrett, 1973) suggested that it was more meaningful to subdivide neonatal disease according to the age at onset. 'Early onset' disease, occurring within the first 10 days of life, and 'late onset' disease occurring usually after 10 days. The early-onset disease, mostly septicaemic in nature but occasionally meningitic, tended to occur in babies with a low birth weight, was frequently associated with a long interval between rupture of the membranes and birth, and was often fatal even if promptly treated. The same serological type of GBS was also almost invariably present in the

vagina of the mother. In the late-onset infection, usually a meningitis, the birth was usually normal and in the first few weeks of life no apparent clinical symptoms were observed. Mortality was appreciably lower if treatment was given early, and the streptococcus was scarcely ever isolated from the mother's vagina.

The apparent seriousness of neonatal disease caused by GBS has prompted a large number of epidemiological surveys to be performed in the last decade with a view to define incidence, sources of infection, and modes of spread of GBS among humans. Howard and McCracken (1974) described the incidence of infection and of mortality caused by GBS and E. coli in neonates in Texas, U.S.A., and found that in the first year of study (1969) streptococcal disease accounted for 1.5 cases of infection per 1,000 live births. During the next three years the incidence of GBS infection declined to approximately 0.5 cases per 1,000 live births, and then in 1973 a significant increase in cases occurred amounting to a frequency of 2.5 cases per 1,000 live births. During the five year study period the incidence of disease due to E. coli remained constant at about 0.5 cases per 1,000 live births. Mortality rates of the infected infants were almost similar for both GBS and E. coli infections. Thirty-one percent of neonates with streptococcal infection and 27% of patients with E. coli died.

In 1977, Ferrieri et al. presented evidence to suggest that neonatal acquisition of GBS at birth was relatively common, and that serious infection by GBS may be as many as one case per 100 colonised infants, with a total infection rate of 2-3 per 1,000

live births. These findings closely agreed with those of Baker and Barrett (1973) and Franciosi et al. (1973).

Recently, total carriage of GBS by neonates in the U.S.A. has been reported as being 11.3% (Maurer et al., 1979) and 12.5% (Pass et al., 1979) and an infection rate of at least three cases per 1,000 live births (Baker, 1977). Some workers suggest, however, that these results are not applicable to the European situation and that infections due to GBS, although of undoubted importance in infants, do not constitute such a serious threat as in the U.S.A.. Early work in Sweden described an incidence of 0.1 cases per 1,000 live births (Bergquist, 1974). In Czechoslovakia, not a single case was found among 1438 babies in 1976 (Jelinkova, 1977). In a London hospital, during a period of five years, seven serious cases of neonatal infection due to GBS were diagnosed from 6,500 deliveries, an incidence of 1.1 cases per 1,000 live births (Finch et al., 1976). In Aberdeen, Reid (1975) found an incidence of 0.7 cases per 1,000 live births in a three year period. The British Communicable Disease Surveillance Centre (1978) calculated a probable infection rate in the U.K. and Republic of Ireland of less than 0.1 cases per 1,000 babies born over a one year period. In Denmark, Kjems et al. (1979) found an incidence of 0.3 cases per 1,000 births in 1977.

The most common sites in neonates from which GBS have been cultured, regardless of whether symptoms were present or not, are the ear, external auditory meatus, nose, anus, umbilicus and throat (Ferrieri et al., 1977; Pass et al., 1979). No significant trend in the serological type of GBS in the different sites has been recognised, but various studies in the U.S.A. have indicated

that neonatal meningitis is predominantly associated with serotype III (Anthony and Concepcion, 1975; Baker and Barrett, 1973; Franciosi et al., 1973; Wilkinson, 1978; and Parker and Stringer, 1979).

In an attempt to obtain a clearer understanding of the pathogenesis and epidemiology of diseases associated with GBS, recent studies have concentrated on determining the principal sources and reservoirs of GBS in humans. The female genital tract has received most attention in the search for the primary site or source of carriage of GBS. Many studies have supported the association of genital tract carriage in pregnant women with subsequent acquisition of the organisms, with or without clinical infection in their newborn infants, within days of birth (Butter and de Moor, 1967; Bergquist, G., 1974; Baker and Barrett, 1973; Anthony et al., 1975). With regard to normal healthy adult females, carriage of GBS in the genital tract has been estimated as ranging from 4.5% to 36% (Parker, 1977). The area swabbed affected results profoundly. Kexel and Beck (1965) reported an isolation rate of 12.1% by swabbing of the vulva, 9.5% vaginal swabbing, and only 6.7% by swabbing the cervix. Methods of sampling and laboratory processing were also found to affect the results significantly.

It is paradoxical however, that in general lower carrier rates were obtained from groups of pregnant and parturient women in comparison to non-pregnant women (Franciosi et al., 1973; Baker and Barrett, 1973; Finch et al., 1976). These workers reported a carriage rate of 5-12% amongst parturient women and Ferrieri et al. (1977) found a rate of 5.6% of women in the third

trimester of pregnancy.

The acquisition of GBS by newborn infants from mothers positive for GBS during labour ranged from 50-70% of individuals (Baker and Barrett, 1973; Anthony et al., 1975; Ferrieri et al., 1977). The view that acquisition of the organisms seen in neonates occurs either in utero as a direct consequence of contamination of the amniotic fluid, or by direct contact with the GBS-colonised birth canal is now generally held. Evidence to support this hypothesis was provided by Ferrieri et al. (1977) who showed that GBS could be isolated from the ear canals of 29 of 31 GBS-positive infants immediately after birth. Parker (1979) while agreeing that colonisation by GBS of many babies is established from organisms present in the genital tract of women, suggested strongly that the tract is not the primary source of streptococcal carriage, and that GBS colonisation initially takes place in the gut, the cervico-vaginal canal acting as a reservoir.

Studies to determine the relevance of the gut as a principal source of GBS have recently been undertaken. Franciosi et al. (1973) recorded an anal carriage rate of 16.8% in female hospital staff, and Badri et al. (1977) observed a rectal carrier rate of 17.9% and a vaginal carriage rate of only 10.2% in pregnant women. Christensen and Christensen (1979) however, refuted the earlier claims of Parker as to the importance of the gut and concluded from their studies that the urethra was the main human reservoir for GBS.

The upper respiratory tract has also been studied to determine its role as a possible source of GBS in humans. Traditionally,

beta-haemolytic streptococci from the throat were normally casually identified as Lancefield's group A, but as long ago as 1947 the Commission on Acute Respiratory Diseases in the U.S.A. described six patients in a group of soldiers who were suffering from acute upper respiratory tract disease attributable to GBS. The overall carriage rate of GBS by this group of individuals suffering from respiratory infections was 0.2%, in comparison to a healthy control group who had a rate of 0.1%. In another early study carried out in England, Hare (1935) reported a GBS carrier rate of 5% in the throats of nurses and schoolboys. During a field trial in the U.S.A. comparing the efficiency of filter paper strips and dacron swabs in the isolation of beta-haemolytic streptococci, the throats of a large group of children were sampled (Hollinger and Rantz, 1959). Eighty two percent of all streptococcal isolates were classified as group A streptococci. The remaining positive cultures were streptococci of Lancefield's groups B, C or G but further differentiation was not performed. In a similar study with schoolchildren, Nicholas and Steele (1962) observed a 16.6% carriage rate of group A streptococci in the throat and a rate of 7.3% for combined total of groups B, C, F and G streptococci. Ferrieri and Blair (1977) observed a 12% pharyngeal carriage rate of GBS amongst a group of 408 young adults between the ages 16-32 years. Furthermore, they reported no significant difference in carriage between healthy individuals and those suffering from respiratory symptoms. Christensen and Christensen (1979) reported that 7% of pregnant women harboured GBS in the throat, and both Knox (1979) and Chretien et al. (1979) recorded an identical GBS

pharyngeal carriage rate in 4.4% of adults tested. Slack and Mayon-White (1978) found that aspirated material from the nose and mouth of neonates was normally sterile, but in 1% of 400 babies GBS were cultured. Subsequent examinations of the four positive babies indicated that three remained free of clinical infection while one child developed meningitis within 24h of birth. Type Ib GBS was isolated from blood, cerebrospinal fluid, nose, pharynx, skin and rectum of the baby and from the mother's vagina.

Parker (1979) commented that on certain occasions it was possible that acquisition of GBS by neonates may occur after birth. Several reports have presented some evidence suggesting that maternal streptococci may be transmitted to an infant via infected milk during breast feeding (Kenny and Zedd, 1977; Schreiner et al., 1977; Lucas and Roberts, 1978). In each of the cases described, the mother's vaginal swab was negative but GBS were grown from the milk. Two of the mothers had definite clinical signs of mastitis. Butter and de Moor (1967) however, thought that the most likely explanation of streptococcal isolations from expressed breast milk was the earlier contamination of breasts by GBS harboured by the nursing infants.

Other sources of GBS implicated in the pathogenesis of neonatal infection were reported by Steere et al. (1975) and Anthony et al. (1978) who suggested that in many cases of late-onset meningitis the infecting streptococcus was acquired from other GBS-positive infants in the hospital environment. Paredes et al. (1977) surmised that nosocomial spread of GBS among infants

was due to direct transferance of organisms from one baby to another, rather than from staff to babies. Christensen et al. (1979) described an epidemic spread of GBS, type Ib, in the upper respiratory tract of both children and staff at a day-care nursery school. Although contaminated food or milk was ruled out as the vehicle of spread, no firm conclusions were made as to the method of spread of GBS.

Since the work of El Ghoroury (1950) and Haug (1972) showing that animal strains of GBS were different in both biochemical and serological aspects to strains colonising humans, the theory of animal (including infected milk) to man spread of GBS can now be totally discounted.

2) Sampling techniques and laboratory methodology in the isolation of GBS from humans

In the first part of the introduction of this chapter attention was drawn to the clinical significance of neonatal disease due to GBS. A significant proportion of the information now available relating to the pathogenesis of this disease has been obtained by many workers performing epidemiological surveys in various population groups. Throughout these reports, each of the workers have indicated the importance of using a sampling technique from which a meaningful result can be presented. Chapter 2 of this thesis described a series of laboratory tests to determine the relative efficiencies of various swabs and transport media in the recovery of streptococci in the laboratory. The results demonstrate that major differences in recovery of these organisms may occur with different swabs and transport

media. Schauf and Hlaing, 1976 calculated, for example, that counts of GBS in the vagina of pregnant women vary over a wide range (10^1 to 10^5 organisms), and the median number removed by swabbing the vaginal fornix was 550 colony-forming units. Thus, it is clearly apparent that many carriers would simply be missed if certain types of swab were used.

Once the sample has been taken and the swab has been sent to the laboratory a number of different techniques are available for processing of the sample. The addition of chemical compounds and antibiotics to bacteriological media to selectively inhibit certain species of bacteria and thus select out other organisms in culture has been common practice for many years. Lilley and Brewer (1953) reported the use of phenylethyl alcohol in agar for the selective isolation of Gram-positive organisms, and Lowbury and Lilly (1955) added neomycin to Nagler plates to facilitate the isolation of Clostridium perfringens in primary cultures. Elston (1965) described a medium designed specifically to select streptococci (containing 5 mg of neomycin sulphate in 100 ml blood agar). Blanchette and Lawrence (1967) also recommended the neomycin blood agar medium for isolation of streptococci but preferred to use a concentration of 30µg/ml of neomycin. Kidson (1967) described a new selective medium for Streptococcus pyogenes containing polymyxin B sulphate, neomycin sulphate, and fusidic acid in blood agar. This medium was said to be less inhibitory to streptococci than Elston's medium and increased the numbers of streptococcal isolates detected. In 1968, Cloutier-Lambin and Gauvreau reported that the addition of nalidixic acid, a

compound synthesised earlier by Lesher et al. (1962), to blood agar was effective in selecting out beta-haemolytic streptococci in mixed throat cultures. Combination of nalidixic acid (15µg/ml) and neomycin (30µg/ml) in a blood-agar base resulted in yet another medium highly selective for streptococci (Vincent et al., 1971). Black and van Buskirk (1973) claimed that blood agar with gentamicin 5.5µg/ml) was eminently suitable as a general purpose selective medium for beta-haemolytic streptococci, Bacteroides, clostridia, and yeasts. Ferrieri et al. (1977) used 6% sheep-blood agar containing colistin (10µg/ml) and nalidixic acid (15µg/ml) in the first part of a survey to detect GBS in neonates. In the second part of the survey a liquid broth medium consisting of Todd-Hewitt broth with gentamicin (8µg/ml) and nalidixic acid (15µg/ml) into which swabs were placed directly and incubated was used. The results obtained with both methods were judged to be equally satisfactory in the isolation of GBS. Ross (in press) recommended a selective broth medium based on that of Ferrieri's but with neomycin substituted for gentamicin which may inhibit some strains of GBS at the concentration used. Beta-haemolytic streptococci from mixed cultures are usually identified by the haemolytic pattern produced on blood agar (Brown, 1919) and by Gram-stain. Definitive classification into Lancefield's groups (Lancefield, 1933) is performed by serological techniques, but a presumptive identification can be made on the basis of certain biochemical differences between the various serological groups. A number of these presumptive tests are now commonly used in the bacteriology laboratories.

(1) Sensitivity to bacitracin. It was first noticed by Maxted (1953) that streptococci of Lancefield's group A are inhibited by the antibiotic bacitracin. Strips of filter paper were dipped into a solution of bacitracin (5 units/ml) in glycine-phosphate buffer, dried and then placed on the surface of blood agar in the area of expected bacterial growth. Plates were then incubated overnight at 37°C and sensitivity noted. In the subsequent trials 97.5% of 851 group A strains proved to be sensitive to bacitracin, and only 1.7% of 2,386 bacitracin-sensitive strains were not group A streptococci. Updyke (1956) however warned that further evaluation and standardisation of the bacitracin test for group A streptococci should be carried out before complete acceptance was given. Pollock and Dahlgren (1973) examined 4,968 streptococcal strains and found that only 0.5% of group A isolates were resistant to bacitracin, while 2.6% of group B, 6.0% of group C, and 8.0% of group G streptococci were sensitive. A number of recent reports (Petran, 1964; Ederer et al., 1972; Arvilommi, 1976; Coleman et al., 1977; Stoner, 1978) indicated that greater reliability of the bacitracin test was achieved with a semi-standardised bacterial inoculum, and by taking a zone of inhibition 10mm or more around a bacitracin disk (0.04-U) as being indicative of group A streptococcus.

(2) The hippurate test. The ability of GBS alone to hydrolyse hippurate with production of benzoates has been used as a presumptive identification test to distinguish this serological group. Ayers and Rupp (1922) were the first to show that haemolytic streptococci of bovine origin could be differentiated from those of human origin on the basis of hippurate hydrolysis

by the former. The method used was to add ferric chloride to the growth medium, an insoluble precipitate remained if the hippurate in the medium had been split into benzoate and glycol, whereas the mixture became clear on standing five or 10 minutes if hydrolysis had not occurred.

In 1975, Hwang and Ederer improved the hippurate test by detecting one of the hydrolysis products, glycine, by ninhydrim solution. This modification enabled the test to be completed within 2h. All GBS strains produced a positive result whereas all strains of other serological groups were negative. Edberg and Samuels (1976) developed the test further by devising a rapid colourometric indicator system for determination of hippurate hydrolysis by GBS. Hydrolysis was detected by the addition of Rhodamine B and uranium acetate to a broth culture containing 1% sodium hippurate. A dark pink colour indicated hydrolysis, and no colour change indicated no hydrolysis. Complete agreement in the detection of GBS between this method and serological grouping of strains was reported.

(3) The CAMP reaction. This test was named after the authors, Christie, Atkins and Munch-Peterson (1944) and enables the presumptive identification of GBS. The test is based on the ability of GBS to produce a factor that acts synergistically with staphylococcal beta-haemolysin on sheep or ox erythrocytes to produce a zone of complete haemolysis. Under anaerobic conditions this haemolytic effect was considerably more marked. Esseveld et al. (1958) inoculated streptococci at an angle of 35° to 45° to a streak of a beta-toxin producing staphylococcus on sheep-blood agar plates. They noted areas of haemolysis shaped line 'candle-flames' in a positive CAMP reaction after incubation

at 37° for 24h. They reported that 99.5% of GBS and 80% of group A streptococci were positive. Wilson and Miles (1964) reported that it was best to incubate the test plates anaerobically, but added that since 80% of group A streptococci were positive, the test had little value in differentiating GBS.

The method employed by Darling (1975) in the CAMP test, whereby streptococcal strains to be tested were streaked at right angles to the staphylococcal streak, enabled unequivocal identification of GBS. No false positive results with strains of other serological groups were recorded. The results of Wilkinson (1977) confirmed the value of the CAMP test in identifying GBS when performed by the method of Darling.

(4) The pigmentation test. Production of pigment by streptococcal colonies was first described by Orla-Jensen in 1919. He observed that red colouration was produced by several strains of pathogenic streptococci in casein peptone agar and also that 'Streptococcus mastidis' (now GBS) formed an orange colour in casein peptone broth with added soluble starch. Durand and Giraud (1923) noted the importance of starch in the media and maintainance of anaerobic conditions as factors necessary for the production of pigment. In 1934, Lancefield described studies on a strain of GBS which formed a yellow-brown pigment. Plummer (1941) found that 128 of 187 strains of GBS produced pigment, and Esseveld et al. (1958) made a general reference to pigmented GBS colonies.

It was not until 1975 that Fallon proposed that the production of orange or brown pigmented colonies, on starch-containing agar, incubated in an atmosphere of hydrogen and carbon dioxide, could be used for the rapid presumptive identification of GBS. Fallon

recommended Columbia blood agar, which contains 0.1% starch, on which he found that 85% of GBS were pigmented. The use of gonococcal (GC) base medium supplemented with Proteose Peptone for detection of GBS pigment was suggested by Merritt and Jacobs (1976) since the pigment could be observed more easily against the white background of the medium.

In 1977, Islam described an improved medium consisting of starch and serum agar. Stab cultures of GBS in this medium enabled the bacteria to produce a deep orange/red pigment, more intense than that observed on Columbia agar. Almost 80% of GBS tested on this medium were easily identified by their pigment production. Strains of other serological groups were all negative. Also in 1977, Haug and Søderlund warned that GBS grown in contact with trypsin were unable to develop pigment.

Jokipii and Jokipii (1976) compared the performance of three presumptive identification tests for GBS using 371 clinical isolates. They found that hippurate was hydrolysed by 96.1%, the CAMP reaction was positive in 90.5%, and pigment was produced by 97.3% of the strains. A combination of any two tests would have detected over 99.8% of strains.

(5) Tolerance to 6.5% sodium chloride. Bergey's Manual of Determinative Bacteriology (1957) describes the ability of GBS cells to grow in a nutrient broth containing 4.0% NaCl, but not usually in 6.5% NaCl. There is little mention of this property in current literature, however until Facklam (undated), referred to the tolerance of GBS to 6.5% NaCl in broth and suggested that this was a useful property by which GBS could be distinguished from streptococci of Lancefield's groups A, C and G. Braunstein

et al. (1969) using a small number of GBS strains found that growth was possible in 6.5% NaCl, but admitted that the number of cases studied were too few to justify any firm conclusions.

Serological identification of beta-haemolytic streptococci

This subject is reviewed fully in Chapter 4 of this thesis.

AIMS OF THE PRESENT STUDY

The purposes of this investigation included,

(1) to ascertain the prevalence of streptococcal carriage, particularly group B, in the nose and throat of a group of schoolchildren residing in the central Edinburgh area.

(2) to assess the importance of the throat as a primary source or reservoir of GBS.

(3) to compare the efficiency of a number of sampling and laboratory techniques in the isolation and identification of GBS.

Materials and methods

The population

Cultures were obtained from 416 schoolchildren between the ages of five and 18 years, during the months from December, 1979 to April, 1980 inclusive. Classes of approximately 20 children were randomly selected from three primary schools and one senior school in the central Edinburgh area. All social and economic levels were represented, but the majority of children belonged to the Registrar General's Social Groups iii, iv and v. Informed parental consent was obtained for each child sampled.

The culture technique

Sampling of children was performed in the mornings only at the school premises. Three cultures were taken from each child, comprising two pharyngeal swabs, and one swab of the left and right anterior nares. Saliva samples were collected from every fifth child by directing the individual to spit approximately 1 ml of saliva into a sterile glass McCartney bottle.

To obtain throat swabs, each child was seated and instructed to raise the head and look at a distant point on the ceiling. A wooden spatula was used to depress the tongue and the swab was gently applied to the throat, starting at the right tonsillar area and moving onto the right arch, following the contour of the uvula and progressing to the left arch and left tonsil. The swab was constantly rotated throughout the procedure to ensure equal loading of organisms onto the swab material. During the clinical examinations the presence or absence of tonsils were noted.

After completion of swabbing, children were questioned to ascertain any recent history of sore throats. Those individuals who recalled suffering from a sore throat or dry cough within the previous month were asked to describe the form of treatment received. Any 'tablets' or 'bottles' prescribed by their practitioners were assumed to be 'antibiotics', whereas 'medicines' from a bottle given by the parent were considered to be a 'cough linctus' not containing antibiotic.

Swabs

Buffered, absorbent cotton wool swabs were obtained from Exogen Ltd, Glasgow, U.K., and were used to collect nasal and pharyngeal samples. The nasal swab and one of the throat swabs were placed into their sterile plastic containers immediately after sampling. The other throat swab was immersed into 5 ml of clear Amies' transport medium in a similar plastic container. The swabs were transported to the laboratory for processing within 2h of the samples being taken.

Laboratory processing of swabs

On arrival at the laboratory samples were transferred from the swab to the solid growth media, 5-10% human blood agar containing 1 in 1,000,000 crystal violet. The swab was gently smeared across a small area of media to form a well, from which streaks were made with a bacteriological loop to cover half the plate. Plates were incubated in an anaerobic gas jar at 37°C for 24h. Each swab was then broken off into 5 ml amounts of selective broth media contained in glass screw-capped vials and incubated

overnight at 37°C. The selective broth chosen to aid recovery of streptococci consisted of Todd-Hewitt broth with 5% (v/v) human blood, 15µg/ml of nalidixic acid and 10µg/ml of polymyxin. One drop (0.02 ml) of the overnight broth culture was taken and streaked out onto half plates of 5-10% human blood agar containing 1 in 1,000,000 crystal violet. The plates were incubated anaerobically overnight at 37°C as before.

Laboratory processing of saliva

One drop (0.02 ml) of the collected saliva was streaked out onto half plates of 5-10% human blood agar with 1 in 1,000,000 crystal violet and incubated for 24h at 37°C.

Differentiation of beta-haemolytic bacteria from primary growth plates

After incubation crystal violet blood agar plates were examined visually for areas of beta-haemolysis around bacterial colonies. The colonies suspected of producing this type of haemolytic pattern were picked from the plates and cultured onto standard 5-10% blood agar plates followed by aerobic incubation overnight at 37°C. This process was repeated until a pure growth of colonies exhibiting beta-haemolysis on blood agar was obtained.

Presumptive identification of streptococci

A battery of tests, the principles of which have been described in the introduction to this chapter, was used to presumptively identify streptococci picked from the primary growth plates.

(1) Gram-staining of bacteria was performed to identify any non Gram-positive cocci.

(2) Lancefield's group A streptococcus was presumptively identified by its sensitivity to bacitracin. Bacteria were streaked out onto 5-10% human blood agar plates. At the end of the first set of streaks and place of origin of the second streaks a bacitracin disc (0.02 μm, BBL) was placed. Plates were incubated aerobically overnight at 37°C and then examined. Any zone of inhibition of bacterial growth extending beyond the edge of the disk was considered sensitive.

(3) The CAMP test was performed essentially as described by the original authors (Christie et al., 1944) to identify group B streptococci. Plates were prepared with trypticase soya agar (Oxoid Ltd, London, U.K.) with 5% (v/v) sheep blood added. One colony of the Oxford strain of staphylococcus (NCTC 1803) was streaked across the diameter of the sheep blood agar plate. The streptococcal strains to be identified were streaked across one quadrant of the plate perpendicular to the staphylococcal streak. Care was taken to leave a space of a few millimetres between the staphylococcal and streptococcal streaks. Plates were incubated aerobically overnight at 37°C. Presumptive identification of group B streptococcus was made when an arrowhead area of haemolysis in the media at the junction of the two streaks was observed.

(4) Pigmentation tests were performed according to the method described by Merritt et al. (1976) and was used as another method of identification of group B streptococci. Bacteria picked from a blood agar plate using a straight nickel wire, were directly stabbed to a depth of approximately 1cm into 5 ml of Columbia agar (Oxoid Ltd, London, U.K.) contained in a glass tube. The tubes were incubated aerobically for a period up to

three days at 37°C, after which examination for pigment was made. A presumptive identification of group B streptococcus was made by observation of a red or orange pigment which was clearly visible in the medium extending from the stab.

(5) The ability of bacteria of streptococcal groups A, B, C and G to grow in the presence of various concentrations of sodium chloride in Todd-Hewitt broth was examined. Tubes containing 5 ml of the broth with final salt concentrations ranging from 0.5% (w/v NaCl) and increasing by graduations of 0.5% NaCl to a final concentration of 10% (w/v NaCl) in broth were prepared. An additional 15 strains of group B streptococci for this experiment were obtained from the Royal Infirmary of Edinburgh. The bacteria were incubated overnight at 37°C in 10 ml of Todd-Hewitt broth, harvested by centrifugation and resuspended in standard nutrient broth (Oxoid Ltd, London, U.K.). One drop (0.02 ml) of a 10^5 dilution of the original culture in nutrient broth was dispensed into each of the tubes containing Todd-Hewitt broth with added sodium chloride. The tubes were incubated overnight at 37°C. Bacterial growth in the broth was assessed visually. From the last two tubes showing growth and the remaining tubes with no visible growth, 0.02 ml of broth was taken and plated onto blood agar. The plates were incubated overnight at 37°C and then examined for bacterial colonies.

Serological identification of streptococci

Beta-haemolytic streptococci were serologically grouped using a commercially available coagglutination technique, the Streptosec test (Organon Teknika Ltd, St. Neots, U.K.). Fifty

test slides with four reaction areas marked A, B, C and G corresponding to the streptococcal groups were supplied with each kit. Dried reagents consisting of antisera against groups A, B, C and G bound to killed staphylococcal cells were contained within the lettered reaction areas.

Test strains of streptococci were incubated in 2 ml of Todd-Hewitt broth overnight at 37°C. One drop (0.02 ml) of the bacterial suspension in broth was pipetted onto each of the four reaction areas and mixed with the dried reagents. The slide was gently rocked for up to 5 min and coagglutination reactions were noted.

Further aspects of the Streptosec coagglutination technique are presented in Chapter 4.

Serological typing of group B streptococci (GBS)

Strains of streptococci identified as belonging to Lancefield's serological group B were further categorised into serotypes Ia, Ib, Ic, II and III on the basis of their type-specific surface antigens. The antigens were prepared by the method of Lancefield (1934). Bacteria were cultured in 50 ml of Todd-Hewitt broth by incubation overnight at 37°C. Cells were harvested by centrifugation at 1,000g for 15 min and washed twice in successive volumes (10 ml) of saline (0.85% w/v NaCl). The type-specific antigens were extracted from bacteria by heating the cells in 0.2M HCl at 50°C for 2h. The cells were discarded by centrifugation at 1,000g and the supernatant fluid was neutralised by 0.2M NaOH, and stored at 4°C until use.

Serological identification was performed by double diffusion in agar gel (Freimer, 1963) of the extracted antigens with type-specific antisera. Precipitin lines in the gel were read after the reaction had been allowed to proceed for 4h.

Detailed descriptions of the double diffusion test and the methods used to obtain type-specific group B antisera are described in Chapter 4.

Statistical analysis

Where appropriate, results were analysed using the chi-square test, expressed by the formula:

$$Chi^2 = \frac{N(ad-bc)^2}{(a+b)(c+d)(a+c)(b+d)}$$

where N = total number in the sample, and a, b, c, d are the experimental values to be compared.

Chi^2 values were translated into degrees of significance from the abridged chi^2 table of Pearson and Hartley (1954) at one degree of freedom.

Results

The prevalence of beta-haemolytic streptococci in the nose and throat of a random sample of schoolchildren at four schools in the central Edinburgh area is illustrated in Table 3.1. Of the 416 children cultured, a total of 78 individuals, or almost 19% of the population, were found to be harbouring beta-haemolytic streptococci. The majority of positive cultures (76) were obtained from the children's throats, whereas streptococci appeared in nasal cultures of only five individuals, three of whom also carried the organisms in the throat.

In Table 3.2 the carrier rates of beta-haemolytic streptococci in males and females in the population of children studied are compared. Although 7% more females than males were shown to harbour the organisms, statistical analysis of the results by the chi-square test revealed that this was not a significant difference.

Table 3.3 presents a more detailed analysis of children who were carriers of beta-haemolytic streptococci. In addition to analysis of results for both males and females, children were divided into different age groups. The first group (5-8 years) were primary school children, the second group of children (9-12 years) comprised those individuals either in the last few years at junior school or in the first years of senior school; the third group (13-18 years) comprised children at the senior school.

Considering the 5-8 year old group of children first, carrier rates of beta-haemolytic streptococci amongst males and females were around 20% of the total number of children sampled. Taking the difference in sample sizes of males and females of this group

into consideration, no statistical difference in carriage rate was shown.

In the 9-12 year age group the percentage of females carrying beta-haemolytic streptococci was more than twice that recorded for males (29% females, 13% males). Statistical analysis of these results concluded that this was indeed a significant difference ($p < 0.001$). The overall carriage rate for both males and females of this age group, however, was 21%, the same rate as that found in the 5-8 year age group. In the last group of children between the ages 13-18 years only four carriers of beta-haemolytic streptococci were detected from a total number of 57 children. There was no statistical difference in carriage between males and females, but the overall rate of 7% for both sexes was significantly lower than for the preceding age groups.

Comparison of the recovery of beta-haemolytic streptococci from cotton wool swabs stored after sampling in plastic containers only, and cotton wool swabs immersed in Amies' medium during transit to the laboratory is shown in Table 3.4. Of the 81 strains of beta-haemolytic streptococci isolated, 20 strains were detected solely from the cotton swabs not stored in transport media, while a further 24 strains were isolated from swabs held in Amies' transport media. Thirty-six strains of beta-haemolytic streptococci were isolated from both types of sampling device. Statistical analysis of these recovery rates showed that the use of transport media did not significantly improve recovery of beta-haemolytic streptococci when organisms were held for a few hours on the swab prior to culturing in the laboratory. Culture of saliva samples

from every fifth child produced only one positive isolation of beta-haemolytic streptococci, but this strain was additional to those detected from the swabs.

Table 3.5 illustrates the results of a comparison of the primary plate technique and selective broth medium in the detection of beta-haemolytic streptococci from swabs and saliva samples. Thirty-seven of the isolates were identified on the primary crystal violet blood agar growth plates only. Incubation of swabs in selective broth and subsequent plating of the broth onto crystal violet blood agar enabled 17 streptococcal strains to be isolated. A further 27 strains were detected by both of the culture techniques. Statistical analysis indicated that the number of beta-haemolytic streptococci isolations from the primary plate method was significantly greater than the number obtained from the selective broth ($p > 0.010$).

Serological identification of the 81 streptococcal strains isolated was performed using the Streptosec coagglutination test (Table 3.6). Three of the strains cultured from the anterior nares belonged to Lancefield's group A, and two were classified as group G streptococci. The majority of beta-haemolytic streptococci (41 strains) from the throats of the children were identified as group A streptococci. In relation to the total number of children surveyed, almost 10% of individuals were thereby shown to be harbouring group A streptococci in their throats. The group G streptococcus was the second most common of the beta-haemolytic streptococci to be present in the throat (16 strains) and nearly 4% of the population were carriers of

this organism. An equal number of streptococci of groups B and C (nine strains) were found in the throats of the children sampled and these organisms each accounted for 2% of the population. Serological typing of the group B strains enabled identification of six isolates of type Ia, one of type Ib and two strains of type Ic.

Table 3.7 shows the range of reactions obtained with the 81 strains of beta-haemolytic streptococci on the basis of three recognised presumptive identification tests. Thirty-seven of the 44 isolates of group A streptococci, serologically grouped by the Streptosec coagglutination test, were sensitive to bacitracin, i.e. 84.1% sensitivity. Group C organisms were the next most sensitive group to bacitracin, and four of the nine strains (44.4%) isolated were found to be sensitive. Inhibition of five strains (27.8%) of group G streptococci by bacitracin occurred and similarly, only one of the nine group B strains were sensitive.

The CAMP tests (Fig. 3.1) and pigmentation tests (Fig. 3.2) successfully identified all nine strains of group B streptococci and no false-positive results with strains of the other serological groups were obtained.

Each child sampled in this study was asked to give information about recent history or presence of a sore throat. Table 3.8 summarises the answers to these questions. Of the children yielding positive cultures for beta-haemolytic streptococci, 16 individuals or 3.9% of the total population indicated they were suffering from a sore throat at the time of sampling. Seven of these children harboured group A streptococci in the throat,

one child yielded group B, three children had group C, and a further five individuals were culture-positive for group G streptococci. The remaining beta-haemolytic streptococci-positive children (62) did not complain of the presence of sore throat when questioned.

Fifty-five children who were culture-negative for beta-haemolytic streptococci also claimed to be suffering from a sore throat. This number of children comprised 13.2% of the total population. However, after statistical adjustment of the relative sample sizes of carriers and non-carriers of beta-haemolytic streptococci suffering from sore throats, analysis by the chi-square test indicated that no significant difference existed between these two groups.

During the sampling procedure it was noted that approximately 90% of the population had tonsils present.

Tolerance of beta-haemolytic streptococci to sodium chloride in broth

Visible growth of three strains of group A streptococci in Todd-Hewitt broth containing 6.5% NaCl was detected. The remaining 10 strains of group A tested produced visible growth in broth containing 5.5% NaCl. Thirteen of 24 group B strains grew visibly in broth with 6.5% NaCl while the other 11 strains grew in broth with 5.5% NaCl. A smaller number of representatives of groups C and G streptococci were tested and of these strains, four group C strains grew in broth with 6.5% NaCl and a further four strains grew in a concentration of 5.5% NaCl in broth. Two of the group G isolates were observed growing in broth with 6.5% NaCl and the remaining four strains grew in the presence of 5.5% NaCl.

Those bacteria of the four serological groups tested which did not grow in broth containing 6.5% NaCl all produced colonies on blood agar when samples (0.02 ml) from the 6.5% NaCl broth were plated out.

Table 3.1 Numbers and percentage of 416 children who harboured beta-haemolytic streptococci in the anterior nares and throat.

	Non-carriers		Carriers	
	Number	Percentage	Number	Percentage
Total children	338	81.2	72	18.8
Nose	411	98.8	5	1.2
Throat	340	81.7	76	18.3

Note: Three children carried streptococci in both anterior nares and throat.

Table 3.2 Sex distribution of children harbouring beta-haemolytic streptococci.

	Non-carriers		Carriers	
	Number	Percentage	Number	Percentage
Male	186	84.5	34	15.5
Female	152	77.5	44	22.5

Table 3.3 Age and sex distribution of children harbouring beta-haemolytic streptococci.

Age groups	Males			Females			Both		
	NC	C	%	NC	C	%	NC	C	%
5-8 years	87	21	19.4	72	20	21.7	159	41	20.5
9-12 years	70	10	12.5	56	23	29.1	126	33	20.8
13-18 years	29	3	9.4	24	1	4.0	53	4	7.0

NC = non-carriers

C = carriers

Table 3.4 Recovery of beta-haemolytic streptococci from plain cotton swabs with and without transport media.

	Number of isolates
Plain swabs (P) only	20
Swabs in transport medium (T) only	24
P + T	36

Table 3.5 Comparison of the primary plate technique and selective broth medium in the isolation of beta-haemolytic streptococci.

	Number of isolates
Primary plates (PP) only	37
Selective broth medium (SBM) only	17
PP + SBM	27

Table 3.6 Distribution of Lancefield's groups isolated from the anterior nares and throat from 78 children.

	Group A		Group B		Group C		Group G	
	No.	%	No.	%	No.	%	No.	%
Nose	3	0.7	-	-	-	-	2	0.5
Throat	41	9.9	9	2.2	9	2.2	16	3.8

Note: One streptococcal strain from the throat could not be grouped.

Table 3.7 Comparison of methods for the identification of beta-haemolytic streptococci.

	Group A	Group B	Group C	Group G
All strains serologically grouped (Coagglutination)*	44	9	9	18
Sensitivity to bacitracin	37	1	4	5
CAMP test	-	9	-	-
Pigment test	-	9	-	-

* One strain could not be grouped by coagglutination and was not positive with any presumptive test.

Table 3.8 Prevalence of sore throat at the time of sampling amongst children identified as carriers and non-carriers of beta-haemolytic streptococci.

	Non-carriers	Carriers
No sore throat	283	62
Sore throat	55 (13.2%)	16 (3.9%)

Note: The 16 isolates of beta-haemolytic streptococci comprises 7 group A, 1 group B, 3 group C and 5 group G streptococci.

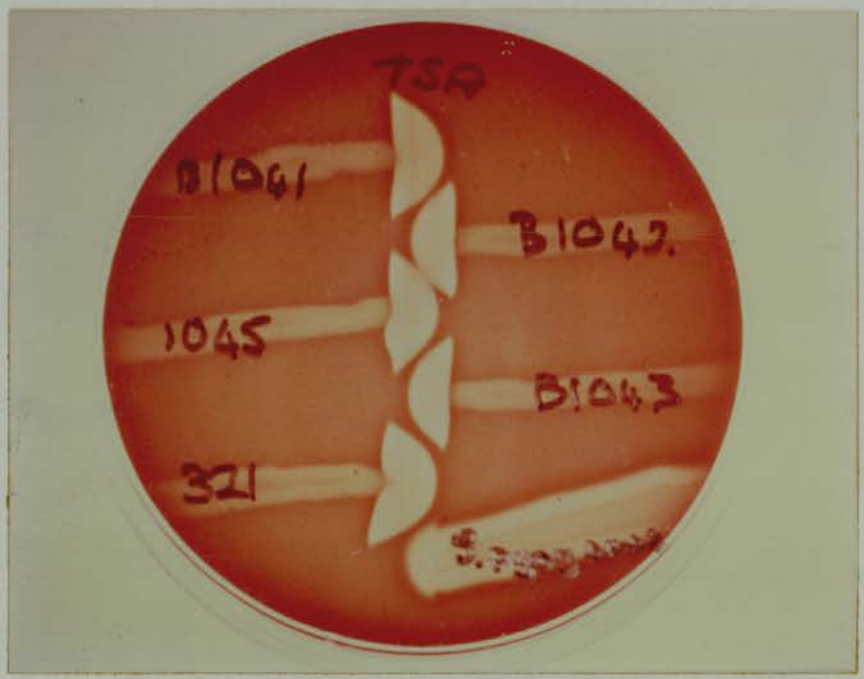


Fig. 3.1 The CAMP test. Typical "arrow-head" areas of haemolysis associated with group B streptococci. Streptococcus pyogenes was included as a control.

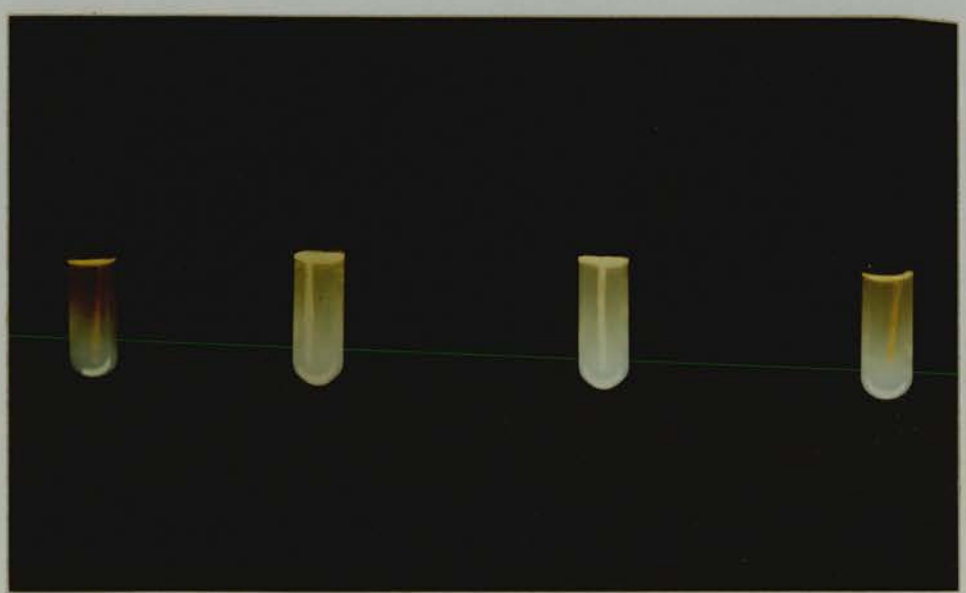


Fig. 3.2 Pigmentation produced by group B streptococci after 48h in Columbia agar (two outer tubes). Streptococcus pyogenes (two strains) were included as controls.

Discussion

The prevalence of beta-haemolytic streptococci in the nose and throat of a group of schoolchildren in Edinburgh was shown to be almost 19% during the months from December to April inclusive. A similar overall carriage rate (23.9%) was reported earlier by Nicholas and Steele (1962), who made no reference to the time of year that the study was performed. Ross (1971) suggested that streptococcal carriage in children varied enormously depending on the time of year. In his study, carriage of beta-haemolytic streptococci in children during the months from October to December was 21%, whereas during April to June the carriage rate fell to 4%.

The present study broadly agrees with the findings of Ferrieri and Blair (1977) in that carriage of beta-haemolytic streptococci did not differ greatly between the sexes with the exception of the middle age group (9-12 years). As expected however, a large difference in carrier rate between younger and older children was found. Almost 21% of children between the ages 5-12 years were shown to be positive carriers of beta-haemolytic streptococci, whereas only 7% of individuals were culture positive in the older age group. The decrease in the prevalence of beta-haemolytic streptococci with increase in age has also been demonstrated by a number of earlier workers (Saslaw and Jablon, 1960; Nicholas and Steele, 1962; Ross, 1971). The reasons for this are not yet clear, but is probably due to development of specific immunity to an ever-increasing number of bacterial types encountered throughout life.

Differentiation of the beta-haemolytic streptococci strains

into Lancefield's serological groups indicated that streptococci belonging to Lancefield's group A (Strept. pyogenes) predominated over the other groups. Most of the group A strains originated from the throat and in only three cases were the bacteria isolated from the anterior nares. Group G organisms were the next most common of the beta-haemolytic streptococci isolated, again mainly from the throat. Only nine strains of each of groups B and C were detected, exclusively from the throat. The overall group B carriage rate (2%) in the 416 children sampled is low in comparison to the results of Ferrieri and Blair (1977) in the U.S.A., but is essentially similar to the results of European surveys (Hare, 1935; Slack and Mayon-White, 1978; Christensen and Christensen, 1979). Further surveys in both the U.S.A. and Europe should be undertaken before definite conclusions are made, but it is possible that a genuine difference in rate of carriage of group B streptococci exists between the populations of the two continents.

The question of whether the throat should be regarded as a significant source of GBS was partly answered by Ferrieri and Blair (1977). They suggested that judging by the results of their survey, the throat was an important reservoir for these bacteria, although the frequency of carriage at this site was less than that found in the genito-urinary tract or gastrointestinal tract. The results of the present study indicate, however, that in children residing in Edinburgh, an overall group B streptococcal carriage rate of 2% does not constitute a truly significant source or reservoir for these organisms. Furthermore, although one case of sore throat was associated with carriage of group B streptococci,

this study was not able to define a direct link between colonisation and clinical infection by group B in the throat.

A number of facts emerged from the comparative tests used in the sampling procedure and laboratory methodology. A number of swabs and transport media were evaluated (see Chapter 2). From the results of this study it was decided to use cotton wool swabs throughout the epidemiological survey. The most promising of the transport media in the laboratory tests was Amies' medium which was used to store some of the swabs during transit to the laboratory. The recovery of beta-haemolytic streptococci from swabs stored in transport medium was not significantly greater from swabs not stored in transport medium. In view of the additional cost of swabs with transport medium it is recommended that plain cotton wool swabs are most suitable for field studies involving streptococci. It is worth noting that some beta-haemolytic streptococci strains from a particular individual were only detected from one of the swabs and not the other. The reasons for this remain unclear, but one explanation may be the apparent random nature of swabbing. It would have been fascinating to have performed yet another series of swabs, immediately after the first series, from the same sites within the same individuals to see if a similar number of positive cultures were obtained.

Comparison of the primary plating technique with selective broth media in the isolation of beta-haemolytic streptococci favoured primary plating. This does not agree with the results of Ferrieri and Blair (1977). In addition, from a purely practical point of view, the primary plates were much clearer and easier to

read than cultures plated from the selective broth.

Serological grouping of beta-haemolytic streptococci is discussed fully in Chapter 4, but in brief, the Streptosec coagglutination test proved to be reliable, rapid and very useful in definitive identification of streptococci.

The specific presumptive tests for beta-haemolytic streptococci (CAMP and pigment) were shown to be as reliable as serological tests and although significantly less costly they were more time consuming. The same can not be said for the bacitracin test. Although 37 of 44 group A strains were successfully identified, a total of 10 strains of groups B, C and G streptococci were also sensitive to bacitracin and these strains would have been wrongly grouped if the test was used in isolation.

The growth tests of beta-haemolytic streptococci in broth containing 6.5% NaCl showed clearly that this test can not differentiate with any degree of certainty between bacteria of the four serological groups.

Chapter 4

Investigations on serological methods of identification of group B streptococci.

Introduction

It is generally accepted that definitive identification of beta-haemolytic streptococci is obtained by serological reaction of specific antigen complexes present in the bacteria, with antisera raised against specific strains of streptococci. Hitchcock in 1924 was the first to demonstrate that most streptococci from human infections possessed a serologically active polysaccharide antigen. Lancefield (1928, 1933) demonstrated that antigens of this class could be extracted from streptococci with hot hydrochloric acid, and purified by alcohol precipitation. These extracts formed precipitates with specific rabbit antisera raised against whole cell suspensions of streptococci. Using this technique Lancefield was able to formulate a classification of beta-haemolytic streptococci, the Lancefield groups. One year later, three serologically distinct types of group B streptococci were identified by hot acid extraction of surface carbohydrates and subsequent precipitation with specific antisera (Lancefield, 1934).

In view of taxonomic reversals and developments in other spheres of bacteriology, it is perhaps surprising but indicative of the quality of the work that Lancefield's classification of beta-haemolytic streptococci is as relevant today as it was at the time of its inception. Furthermore, the original method of extraction of group and type specific antigens from streptococci by heating cells in hot HCl, followed by precipitation with specific antisera, is widely regarded as the standard test by which all other relevant serological methods should be assessed. Disadvantages

of the Lancefield's extraction technique are that the method is time consuming and requires careful handling, particularly in the final stage of neutralisation of the extract by sodium hydroxide (Rantz and Randall, 1955; Noble and Penny, 1974).

A number of other techniques for extraction of group specific carbohydrate antigens from beta-haemolytic streptococci have been suggested:

(1) Fuller's hot formamide technique (Fuller, 1938) is a relatively complex method to extract group antigen by treating cells with formamide for 15 min at 150°C.

(2) Digestion of cells with a proteolytic enzyme extracted from Streptomyces albus (Maxted, 1948).

(3) Extraction of grouping antigen by autoclaving whole cells for 15 min at 121°C (Rantz and Randall, 1955).

(4) Ederer's pronase B enzyme extraction technique (Ederer, et al., 1972) is essentially similar to the method of Maxted. Pronase B is a protease from Streptomyces griseus described by Nomoto and Narohashi (1959).

(5) El Kholy's nitrous acid extraction method (El Kholy et al., 1974) is a simple chemical extraction procedure which involves treating whole cells for 15 min with 4M nitrous acid. The technique does not require the use of heat.

(6) Watson's Streptomyces albus lysozyme enzyme technique (Watson et al., 1975) claims to have an advantage over the other two enzyme extraction techniques in that group D streptococcal antigen can be successfully extracted.

Type specific carbohydrate and protein antigens can be extracted from group B streptococcal cells by heating with HCl for 2h at 50°C (Lancefield, 1934). The other techniques described for extraction of the group antigen are unsuitable as typing methods since protein is destroyed.

Antisera against the group and type antigens can be raised in rabbits by intravenous injection of formalin treated whole cell suspensions (Lancefield, 1934). Detection of antigens by precipitation of antisera with the cell extracts are performed in a variety of ways.

(1) The capillary precipitin test originally described by Swift et al. (1943). Antisera is carefully layered onto a volume of extract in capillary tubes and precipitate forms at the interface of the two liquids.

(2) Double diffusion in agar gel (Ouchterlony, 1949). This technique is described in full in the Methods section of this Chapter.

(3) Counter current immunoelectrophoresis using the Lancefield prepared antigen was adapted by Dajani (1973) for rapid identification of beta-haemolytic streptococci, and by Hill et al. (1975) specifically to identify group B streptococci present within clinical isolates.

More recently, a number of new techniques for the serological grouping of beta-haemolytic streptococci have been developed. Generally, the methods require whole cell preparations of the strains to be tested, and dispense with the need to carry out extensive antigen extraction procedures on the bacteria.

(1) The fluorescent antibody (FA) test has been used extensively in the past, especially in the U.S.A. for grouping streptococci. Moody et al. (1958) were among the first to report on the apparent advantages offered by this technique over the more conventional Lancefield system of grouping. Specific globulin fractions, labelled with fluorescein isocyanate (Coons and Kaplan, 1950), were added to dried smears of a streptococcal suspension and incubated in a moist atmosphere for 30 min. Slides were read with a fluorescence lamp microscope. Improvements to achieve specificity in the technique for the identification of group B streptococci were reported by a number of workers (Tuomi and Nurmi, 1964; Kubin et al., 1968; Smith, 1971; Ederer and Chapman, 1972; Cars et al., 1975).

The fluorescent antibody test has also been used in a few centres to serotype group B streptococci (Romero and Wilkinson, 1974; Bevanger and Maeland, 1977).

(2) The coagglutination technique was adapted for grouping of beta-haemolytic streptococci by Christensen et al. (1973), and has since been developed commercially by a number of manufacturers.

Direct agglutination of untreated streptococcal cells by group-specific antisera is not reliable for two reasons; first, group-specific antigens may not be exposed on the surface of the cell, and secondly, many strains show saline agglutinability (Lancefield, 1933; Rosendal, 1956). It was shown that these problems could be overcome by coating a

certain strain of Staphylococcus aureus with immunoglobulin from specific antisera. A suspension of such staphylococci is agglutinated by the corresponding antigen only, and this reaction is visible to the naked eye. The strain of staphylococcus used was Cowan type 1 which contains a unique cell wall-associated protein molecule, protein A. This protein exhibits a reactivity of high affinity with the Fc portion of IgG (Kronvall and Frommel, 1970), and after combination with anti-streptococcal IgG allows the antigen-combining Fab portion of the immunoglobulin to be directed outwards, away from the cell. In this way, an accessible determinant site for binding specific streptococcal antigen is left exposed on the surface of the staphylococcus.

Christensen et al. (1973) indicated that optimum agglutination was obtained by first incubating streptococcal test strains in trypsin to remove interfering surface protein antigens. Other workers (Arvilommi, 1976; Damask et al., 1979) have reported that trypsin treatment is only necessary if initial results with untreated streptococcal cells are inconclusive.

(3) Agglutination of serum-coated latex particles with streptococcal antigen extracted by the Streptomyces albus enzyme technique has been suggested as a rapid grouping method for beta-haemolytic streptococci (Farrar and Paul, 1979).

The development of numerous techniques to serologically identify beta-haemolytic streptococci has encouraged many comparative studies on these procedures to be performed. A small study (Shukla and

Gupta, 1965), with 55 streptococcal strains showed that group-specific antigens extracted either by the method of Rantz and Randall (1955), and Lancefield's technique (1933) could be identified equally well by reacting against antisera in capillary tubes. Similar findings were reported by Truant et al. (1965) and Hamilton (1972). Noble and Penny (1974) found that in tests with a gel diffusion system the Rantz extraction method allowed more frequent identification of streptococci of groups B and D, in comparison with Lancefield's method. Of the other antigen extraction procedures, Kunter (1965) reported that Fuller's formamide method (1938) was equally as effective in releasing antigen as the Rantz method, and Ederer et al. (1972) preferred to use Pronase B enzyme in grouping tests because of the relative simplicity of the technique over Lancefield's method.

Extraction of antigen by nitrous acid was shown to release a much greater amount of group-specific carbohydrate than other conventional methods, thereby amplifying the precipitin reaction with antisera (El Kholy et al., 1974, 1978).

Assessment of extraction procedures was performed initially by precipitation reactions of group-specific antigens with recognised antisera, either in capillary tubes or in agar gel. Lancaster and Sherris (1960), and Kunter (1965) found the capillary precipitin test to be inferior to the gel diffusion test in serogrouping streptococci. These findings conflicted with those of Noble and Penny (1974), who commented on the lack of reliability with the gel diffusion test.

Recently, most workers have included the coagglutination

technique (described previously) of Christensen et al. (1973) in their comparative tests. Throughout the extensive literature on this subject the general conclusion is that the coagglutination procedure is as reliable as Lancefield's test and is very much easier to perform. Arvilommi (1976) warned of the dangers of non-specific agglutination when using laboratory prepared reagents, but careful interpretation of these reactions enabled false-positive results to be recognised. Later in 1976 the first of the commercial coagglutination kits (Pharmacia Diagnostics, London, UK) for identification of streptococci of groups A, B, C and G became available. In England, Farrell and Amirak (1976) reported that over 90% of strains could be correctly identified in tests with a 4-h broth culture rather than the 24-h culture recommended by the manufacturer. In the U.S.A., Rosner (1977) published confirmatory results. Lim et al. (1979) recommended that increased accuracy of grouping with Pharmacia's test could be achieved by using the supernatant broth in addition to pure culture. Significant improvements to commercial coagglutination kits have been made in the last few years and reports (Koshi et al., 1979; Damask et al., 1979; Easmon et al., 1980; Webb et al., 1980) indicated that a high degree of reliability for grouping streptococci has now been achieved.

Grouping of beta-haemolytic streptococci by the fluorescent antibody test has received less attention in recent years following the successful application of the coagglutination technique. Furthermore, cross-reactions and non-specific reactions with streptococci of groups A, C and G are well known hazards of the

immunofluorescence procedure (Moody et al., 1958; Curtis and Krause, 1964; Karakawa et al., 1965; Franek and Kubin, 1968; Kronvall, 1973). Cars et al. (1975) found that improved specificity could be achieved by using the Fab fragments only of specific IgG labelled with fluorescein isocyanate. In their study, extensive absorption of conjugates was still required to obtain the required specificity. Serotyping of group B streptococci by the immunofluorescence test has, however, been more successful (Cropp et al., 1974). Bevanger and Maeland (1977), classified 84 out of 90 group B strains into serotypes Ia, Ib, II and III. It was thought that non-typable strains probably contained only the X or R protein antigen complexes, against which fluorescent labelled sera were not available. Cross-reactivity amongst strains in this study was eliminated when bacteria were digested with pepsin before testing, but not by trypsin treatment. This result could not be fully explained.

Comparability studies have also been performed to assess the accuracy of the counterimmunoelectrophoresis (CE) technique of Dajani (1973) as a means of rapid identification of beta-haemolytic streptococci. Many workers (Edwards and Larson, 1974; Wadstrom et al., 1974; Hill et al., 1975; Greer et al., 1978) have stated a preference for CE over Lancefield's capillary test, particularly for identification of group B organisms, because of the greater reliability, rapidity and simplicity of the technique. Cropp et al. (1974) compared CE with the fluorescent antibody (FA) test and concluded that the two methods were of equal specificity but in some instances the FA test was more sensitive. Webb et al.

(1980) found that CE was also as sensitive as a commercial coagglutination test in detecting group B streptococcal antigen in cerebrospinal fluid from infants with meningitis. Perhaps the most useful application of the CE technique was described by Fenton and Harper (1978) who identified group B streptococci from mixed cultures in broth after incubation for 20h. This offered considerable advantages when a rapid clinical diagnosis was required.

Enzyme-linked immunosorbent assay (ELISA)

Enzyme-labelled antibodies have been used for some years for the detection of virus antigens in tissue sections (Nakane and Pierce, 1966; Wicker and Avrameous, 1969). Their use in quantitative procedures was made possible by the introduction of an enzyme-linked immunosorbent assay (ELISA). The techniques for ELISA were initially pioneered by Engvall and Perlmann (1971, 1972) and by Van Weemen and Schurs (1971, 1972). An antigen coupled to a solid phase support is incubated with specific antiserum. The enzyme-labelled antiglobulin (conjugate) is then added to the system and becomes attached to the carrier surface. The presence of bound conjugate is denoted by addition of an enzyme substrate which changes colour when it is degraded.

The types of carrier most commonly used are polystyrene beads, tubes or microtitration plates (Engvall and Perlmann, 1972; Voller et al., 1974), but some workers have used Sepharose beads, as these permit covalent linking of certain antigens to the surface (Deelder et al., 1975).

Two types of enzyme for conjugation with immunoglobulin have mainly been used in ELISA. Engvall and Perlmann (1972) favoured alkaline phosphatase as the enzyme marker. It has a high activity, and its substrate is cheap and nontoxic, with a bright yellow reaction colour that can be assessed visually. Horseradish peroxidase was shown long ago (Avrameus and Uriel, 1966; Nakane and Pierce, 1966; Nakane, 1975) to be a good choice of enzyme for conjugation. This also has high activity, is cheap and yields a visible brown/purple reaction product with the substrate.

Since the first description of the technique, ELISA has been used in a wide range of fields . It is, however, in the field of virology and bacteriology that the ELISA system has had the greatest impact. The assay has been exploited with considerable success for both detection of viruses and measurement of specific antibodies in viral diseases caused by measles, cytomegalovirus and rubella viruses (Voller et al., 1975). In addition, plant viruses have also been identified by this method by Voller et al. in 1976 and Clark and Adams in 1977.

Carlsson et al. (1972) were the first to apply the method in bacteriology when they showed that human antibodies to Salmonella 'O' antigen could be accurately measured. Holmgren and Svennerholm (1973) adapted ELISA for detection of antibodies to Vibrio cholerae, and similarly, immunoglobulin levels against Escherichia coli (Jodal et al., 1974) and Brucella (Engvall and Carlsson, 1976) were detected.

Russell et al. (1976) showed that ELISA was specific and highly sensitive for the measurement of antibodies against

streptococcal M protein in human and rabbit sera. Rissing et al. (1978) quantified antibodies against Haemophilus influenzae type B in human sera, and Poxton (1979) who used the technique to differentiate Bacteroides species was among the first to recommend ELISA as a serological method for grouping and typing of bacteria.

At present, binding of either protein or lipopolysaccharide antigen complexes to the polystyrene carrier surfaces has been easily accomplished. Those who attempted to bind pure polysaccharides have met with little success (personal communication, Dr B.M. Gray, Department of Paediatrics and Microbiology, University of Alabama in Birmingham, U.S.A.).

AIMS OF THE PRESENT STUDY

The overall aims of this study were firstly, to examine the possibilities of adapting the ELISA technique for rapid serological identification of streptococci of groups A, B, C, D and G; secondly, if grouping by ELISA became possible, to further modify the assay for serotyping of group B streptococci; and third, to carry out a comparative trial of the developed ELISA technique with double

diffusion in agar using the 'Lancefield' antigen, and a new commercial coagglutination technique (Streptosec, Organon Teknika Ltd, St. Neots, UK) for streptococcal grouping.

Materials and Methods

Bacteria

Reference strains of beta-haemolytic streptococci representative of Lancefield's groups were as follows; group A (NCTC 8198), group C (NCTC 8543), group D (Colindale strain) and group G (NCTC 9603). Reference strains of group B streptococci (GBS) were Ia (090R, 1963), Ia (090R), Ib (H36B), Ic (NCTC 11078), II (NCTC 11079), III (NCTC 11080). Clinical isolates of beta-haemolytic streptococci obtained from patients attending the Royal Infirmary of Edinburgh included: 20 strains of group A, 35 of group B, 15 of group C, 15 of group D and 15 of group G. All strains were maintained on nutrient agar slopes in screw-capped bottles at $4^{o}C$.

Growth of bacteria

Streptococci were routinely cultured in Todd-Hewitt broth (Oxoid Ltd, London) and on 5-10% human blood agar plates.

Preparation of bacterial extracts

Method 1. Group specific carbohydrate antigens were extracted from whole streptococcal cells essentially as described by Lancefield (1933). Each strain was grown in 50 ml of Todd-Hewitt broth for 18h at $37^{o}C$. Cells were harvested and washed in three successive volumes (10 ml) of saline (0.85% w/v NaCl) by centrifugation at 1,000g for 15 min. Antigen was extracted from bacteria by heating the cells in 0.4 ml of 0.067M HCl for 10 min at $100^{o}C$. The suspension was cooled under a running tap and neutralised with 0.5M NaOH. The cells were then discarded

after centrifugation at 1,000g for 15 min. The supernatant fluid, containing antigen carbohydrate, was stored at 4°C until use.

Method 2. Type specific protein and carbohydrate antigens of the GBS serotypes were obtained from whole cells by the method of Lancefield (1934). Cells were cultured for 18h at 37°C in Todd-Hewitt broth and were harvested and washed in saline as before. Antigen was extracted by heating the cells in 0.2M HCl at 50°C for 2h. Cells were discarded and the supernate, neutralised by 0.2M NaOH, was stored at 4°C until use.

Antisera

(1) Grouping sera

Antisera for Lancefield's streptococcal groups A, C, D and G were obtained from Wellcome Reagents Ltd, London. Group B (GBS) antiserum was raised in rabbits in this laboratory. To prepare the vaccine, passaged GBS type Ia strain (090R, 1963) was cultured overnight at 37°C in 250 ml of Todd-Hewitt broth. Cells were harvested by centrifugation at 1,000g for 15 min and the supernate was discarded. The bacteria were washed sequentially in three volumes (25 ml) of saline (0.8% w/v NaCl) by centrifuging at 1,000g for 15 min. To kill bacteria, 3 ml of 20% (v/v) formol saline in 17 ml of saline were added to the cells and after thorough mixing the suspension was left on the bench at room temperature for 3h. Formol saline was removed by centrifugation at 1,000g for 15 min and cells were suspended in 25 ml of saline. The completed vaccine of cells in suspension was stored at 4°C until use. Inoculation schedules are described later.

(2) Typing sera

Antisera for the five main serotypes of GBS were raised in this laboratory against the following reference strains; Ia (090R), Ib (H36B), Ic (NCTC 11078), II (NCTC 11079), III (NCTC 11080). Bacteria were cultured in 250 ml of Todd-Hewitt broth overnight at 37°C. Cells were harvested and washed in three successive volumes of saline (25 ml) by centrifugation at 1,000g for 15 min. Type Ic cells were killed by heating for 30 min at 56°C in a water bath, and the cells were resuspended in 25 ml saline (0.85% NaCl). The vaccine was stored at 4°C until use.

Bacteria of the remaining serotypes were killed by adding 3 ml of 20% (v/v) formol saline in 17 ml of saline to the cells and leaving at room temperature for 3h. Half the fluid volume of the suspension was replaced by saline and the pH was adjusted to two by the addition of 0.5 ml of 0.5M HCl. Cross-reactive protein antigens present on the cell surface were destroyed by incubation of the bacterial suspension overnight at 37°C with 0.15 ml pepsin (5 mg/ml in water) (BDH Chemicals Ltd, Poole, UK). Final neutralisation was performed with 0.5M NaOH, and the vaccines were stored at 4°C until use.

Inoculation schedule

The vaccines prepared to obtain group and type specific GBS antisera were injected intravenously into New Zealand white rabbits via the marginal ear vein. The inoculation schedule was as follows; an initial dose of 0.5 ml of cells in suspension was administered to each rabbit on week one, followed by twice

weekly injections of 1 ml of vaccine for four weeks. Three days after the final inoculation, test bleeds (5 ml) were obtained from each animal and the antibody content of the sera was assessed. This was performed by making doubling dilutions of the sera and observing homologous reactions with group and type specific antigen extracts in capillary precipitation tests. Provided that acceptable antibody titres were obtained, the rabbits were exsanguinated on the next day and the sera, divided into 5 ml amounts, were stored at -20°C until use. Merthiolate (1 in 10,000) was added as a preservative.

Absorption of sera

Cross-reacting antibodies were absorbed from each of the grouping sera by adding sequentially 10 ml of a 1 in 10 dilution of serum in PBS (0.05M sodium phosphate buffer containing 0.15M sodium chloride, pH 7.4) to packed PBS - washed bacteria from 100 ml of overnight culture of the reference strains (groups A, B, C, D and G), and incubating for 30 min at room temperature.

Capillary precipitation tests

The method employed was essentially that described by Wilkinson (1973). Streptococcal antigen extract (0.1 ml) was dispensed into wide ended capillary tubes set into a wedge of plasticene. Approximately 0.1 ml of antisera was then carefully run down the side of the capillary to avoid mixing of extract with antiserum. Precipitation bands at the liquid/liquid interface were read within 10 min.

Enzyme-linked immunosorbent assay

The 'double-sandwich' and 'indirect' techniques of enzyme-linked immunosorbent assay (ELISA), as described by Voller et al. (1976) and Poxton (1979), were utilised and modified throughout the course of this study in an effort to adapt the assay as a suitable procedure for identification of streptococci. General principles of the techniques are described below while specific modifications to the assay are presented in the results section of this chapter.

(1) The indirect ELISA. Fifty microlitres of streptococcal antigen in 0.05M sodium carbonate buffer, pH 9.6, containing 0.02% (w/v) of sodium azide (NaN_3) were added to each well of a disposable flat-bottomed polystyrene microtitration plate (Sterilin Ltd, Teddington, Middlesex). To prevent evaporation of fluid, plates were sealed in aluminium foil and incubated at 37°C for 4h, then held at 4°C overnight to allow adherence of the antigen to the polystyrene surface. The plate was washed three times with 0.15M NaCl containing 0.05% (v/v) Tween 20 by directing the nozzle of a wash-bottle into each well and filling it to the top. The plate was shaken dry after each wash. Specific antiserum, diluted in PBS (0.05M sodium phosphate buffer containing 0.15M NaCl, pH 7.4) containing 0.05% Tween 20 and 0.02% NaN_3 was added to each well (50µl) and incubated at room temperature for 4h. The plate was washed as before and anti-rabbit IgG conjugated to alkaline phosphatase (Miles Laboratories UK Ltd, Slough, UK) and diluted in the same buffer as the

antiserum, was added to each well (50µl) and incubated overnight at room temperature. Again, wells were washed with PBS-Tween, shaken dry, and 50µl of alkaline phosphatase substrate solution, containing p-nitrophenylphosphate (Sigma Chemical Co., London, U.K.) at 1 mg/ml in 0.05M carbonate buffer, pH 9.8 with 0.001M $MgCl_2$, were added to each well. The plate was then left uncovered at room temperature. Results were recorded by visual examination of the plate within 45 min of addition of substrate, a change from colourless to yellow was read as a positive result.

(2) Double sandwich ELISA. This technique is similar to the indirect method already described, but in the first stage of the assay, specific antiserum diluted in 0.05M carbonate coating buffer (50µl) was bound to the polystyrene surfaces of a microtitration plate by incubation for 4h at 37°C. Wells were washed three times with PBS-Tween and the plate was shaken dry. Streptococcal antigen in PBS-Tween (50µl) was then added to each well and the plate was incubated overnight at 6°C. Wells were again washed three times with PBS-Tween. IgG of the same specificity as the coating antiserum and labelled with alkaline phosphatase was diluted in PBS-Tween and 50µl was dispensed into each well. The plate was incubated for 3h at 37°C, washed with PBS-Tween, and 50µl of alkaline phosphatase substrate were added to each well. As before, results were recorded by visual examination of the plate for colour change.

Preparation of IgG-alkaline phosphatase conjugate

The specific IgG-enzyme conjugate prepared in the laboratory was used in the 'double-sandwich' ELISA technique.

(1) Purification of gamma-globulin. A 45% saturation of ammonium sulphate in GBS type Ia antiserum was achieved by combining 4 ml of 1.56M ammonium sulphate solution, 1 ml of water, and 5 ml of serum (Engvall and Perlmann, 1971). The tube was shaken for 1 min and the immunoglobulin precipitate was collected by centrifugation at 1,150g for 40 min at room temperature. The precipitate, in 5 ml of PBS, was dialysed against 2 litres of PBS, pH 7.0, overnight at 4°C. Finally, the total amount of purified immunoglobulin was measured by performing a protein assay (Lowry et al., 1951).

(2) Conjugation of immunoglobulin to alkaline phosphatase. The conjugation of GBS type Ia IgG with alkaline phosphatase was performed by the method of Engvall and Perlmann (1971). Alkaline phosphatase (Sigma type VII), at a concentration of 5 mg/ml in 2.6M ammonium sulphate, was obtained from the Sigma Chemical Company, London, UK. A volume (0.3 ml) of this suspension, containing 1.5 mg protein, was centrifuged at 1,150g for 10 min at 4°C. The supernatant fluid was discarded and the alkaline phosphatase pellet was mixed with 0.1 ml of the purified immunoglobulin in solution (5 mg/ml) containing specific Ia antibody. After dialysis against 2 litres of PBS overnight at 4°C, 4µl of 25% (v/v) glutaraldehyde were added to a final concentration of 0.2%. The solution was left at room temperature for 2h and then

diluted to 1 ml with PBS. To remove excess glutaraldehyde. dialysis against 2 litres of 0.05M Tris-HCl buffer, pH 8.0, was carried out overnight at 4°C. The resultant conjugate was purified by passing the sample through a column (60 x 1.6 cm) of Sepharose 6B (Pharmacia Diagnostics, London, UK) in Tris-HCl buffer, pH 8.0. Enzyme activity of the fractions was assessed by removing 50µl of liquid from each fraction and adding to 50µl of alkaline phosphatase substrate solution. The development of a colour change from colourless to yellow indicated the presence of the enzyme in the fraction. Protein content of each of the fractions was measured by the protein assay of Lowry et al. (1951). By this method those fractions containing conjugate were identified and pooled. Following the addition of 5% serum bovine albumen and 0.2% sodium azide, the conjugate was stored at 4°C until use.

Gel-diffusion tests

Double diffusion in agar gel (BDH Chemicals Ltd, Poole, UK) was performed by a modification of the Ouchterlony method (Freimer, 1963). Acetone rinsed glass microscope slides were covered with 4 ml of 1% (w/v) agar in water. A Feinberg cutter was used to punch out five peripheral circular wells surrounding a central well in the gel (see Fig. 4.1). Each of the outside wells was filled with unknown streptococcal antigen extracts (5µl) prepared by the Lancefield technique, the central well contained specific antiserum. To prevent drying of the gel, slides were stored for up to 6h in an enclosed moist box after which precipitin lines in the gel were recorded.

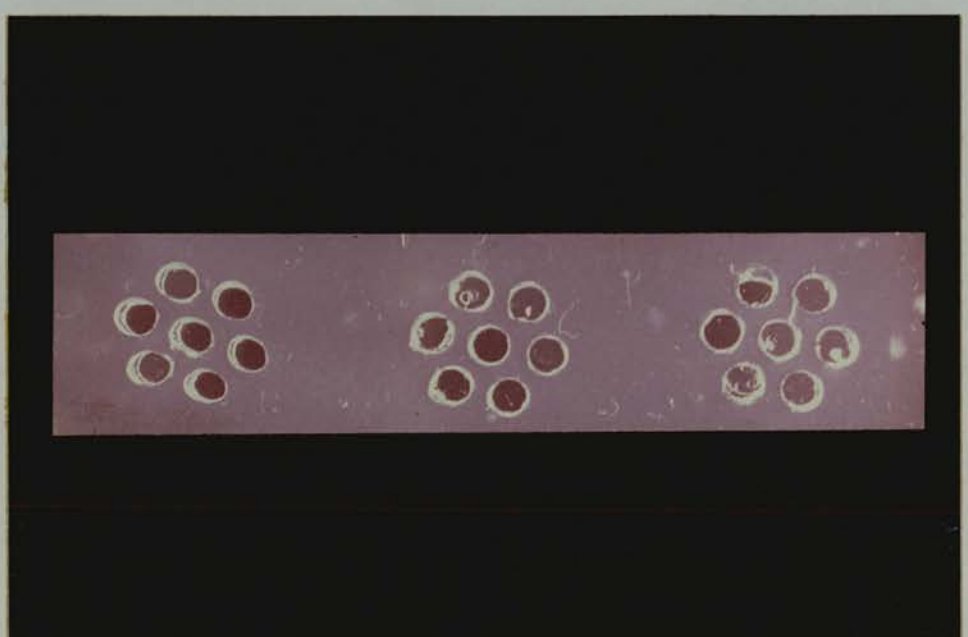


Fig. 4.1 Prepared gel slide used for double diffusion in agar (Modified Ouchterlony method).

Slide co-agglutination tests

Serological differentiation of streptococci of Lancefield's groups A, B, C and G streptococci was carried out using a commercially available test based on the coagglutination technique (Streptosec, Organon teknika Ltd, St. Neots, UK). Kits contained enough reagents for 50 grouping tests and comprised 50 slides, each with four reaction areas marked A, B, C and G, and 200 plastic spatulas. Dried reagents consisting of serum with either anti-A, B, C or G group specific activity bound to the cell surface of heat and formaldehyde treated Cowan-1 staphylococci were contained within the designated reaction areas.

Tests were performed on streptococcal strains that had been cultured aerobically overnight at 37°C on 5-10% human blood agar plates. One colony was picked from the plate, transferred to 2 ml of Todd-Hewitt broth and incubated for 4h at 37°C. One drop (0.02 ml) of the streptococcal suspension was pipetted onto each of the four reaction areas and thoroughly mixed with the dried reagents using a plastic spatula. To avoid contamination a fresh spatula was used for each of the reaction areas. The plate was gently rocked for 2 min and agglutination was noted.

Colonies were also tested directly from the blood agar plate. Cells were evenly suspended in 0.25 ml of Todd-Hewitt broth by vortexing the contents in a tube for 2 min. The suspension was then tested as before.

Results

Section 1 - Antisera

(1) Group B streptococcal antisera. Vaccine comprising group B cells suspended in saline was injected twice weekly into a New Zealand white rabbit over a period of one month. A test bleed taken from the rabbit after this time showed that there had been a strong homologous response by the animal. The serum produced a definite band of precipitation at a dilution of one part serum to seven parts saline with 'Lancefield' extracted group B antigen in the capillary precipitin test (Fig. 4.2), this degree of activity was considered adequate. The rabbit was then exsanguinated, yielding approximately 110 ml of whole blood, from which 50 ml of serum was obtained. Testing of the group B sera against 'Lancefield' antigens prepared from groups A, B, C, D and G streptococcal strains by the capillary precipitin test showed the serum to be specific.

(2) Group B type-specific antisera. Specific antisera for serotyping group B streptococci were obtained from rabbits following an inoculation schedule similar to that used to raise group-specific antisera. At the end of one month test bleeds were taken from each animal and the sera tested by the method described on page 102. Except for one rabbit (No. 3) injected with the type Ib vaccine, all other animals produced sera which at a dilution of 1 in 8 in saline reacted strongly with the specific 'Lancefield' prepared antigen. A test bleed from rabbit No. 3 after four further injections of Ib vaccine over two weeks showed a strong homologous response with specific antigen.

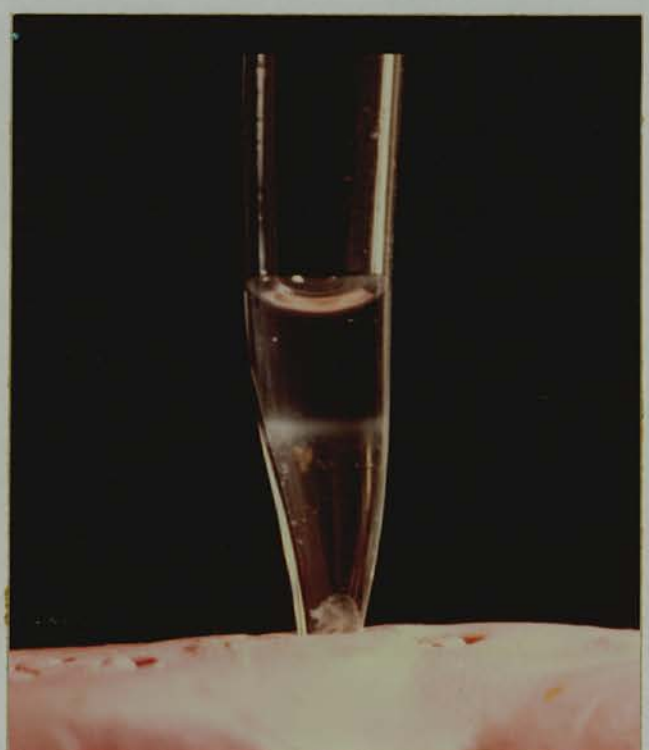


Fig. 4.2 Capillary precipitin test showing band of precipitation at antigen-antibody interface.

All rabbits were exsanguinated and the following approximate volumes of whole blood and sera were collected:

Serotype Ia - 115 ml blood, 50 ml serum

Ib - 90 ml blood, 35 ml serum

Ic - 100 ml blood, 45 ml serum

II - 90 ml blood, 40 ml serum

III - 110 ml blood, 50 ml serum

Specificity of each of the typing sera was initially assessed by performing capillary precipitin tests with 'Lancefield' prepared antigens. Ia serum reacted strongly with Ia antigen and weakly with Ic antigen. Ib serum reacted strongly with Ib antigen only. Ic serum reacted strongly with Ia and Ic antigens and weakly with the Ib antigen. Types II and III sera appeared to be completely specific for their respective antigens.

Section 2 - Precipitation tests in agar gel

Serological identification of the 100 strains of beta-haemolytic streptococci was performed in the first instance by double diffusion of the 'Lancefield' prepared group-specific antigen against antisera in agar gel. All strains tested had been obtained from the diagnostic laboratory of the Bacteriology Department at the Royal Infirmary of Edinburgh and had previously been identified by a variety of methods. The results obtained with the diffusion tests were in complete agreement with those reported from that laboratory.

Antiserum was unabsorbed, undiluted and approximately 5µl was

used for each test. Cross-reactivity between the test antiserum and heterologous strains was observed in the gel tests by minor spur lines in a minority of the tests; at no time did serious confusion arise as to identity of the strains. From the time of seeding the broth with pure culture a minimum of 24h was required to establish identification.

Section 3 - Modification of ELISA technique for grouping streptococci

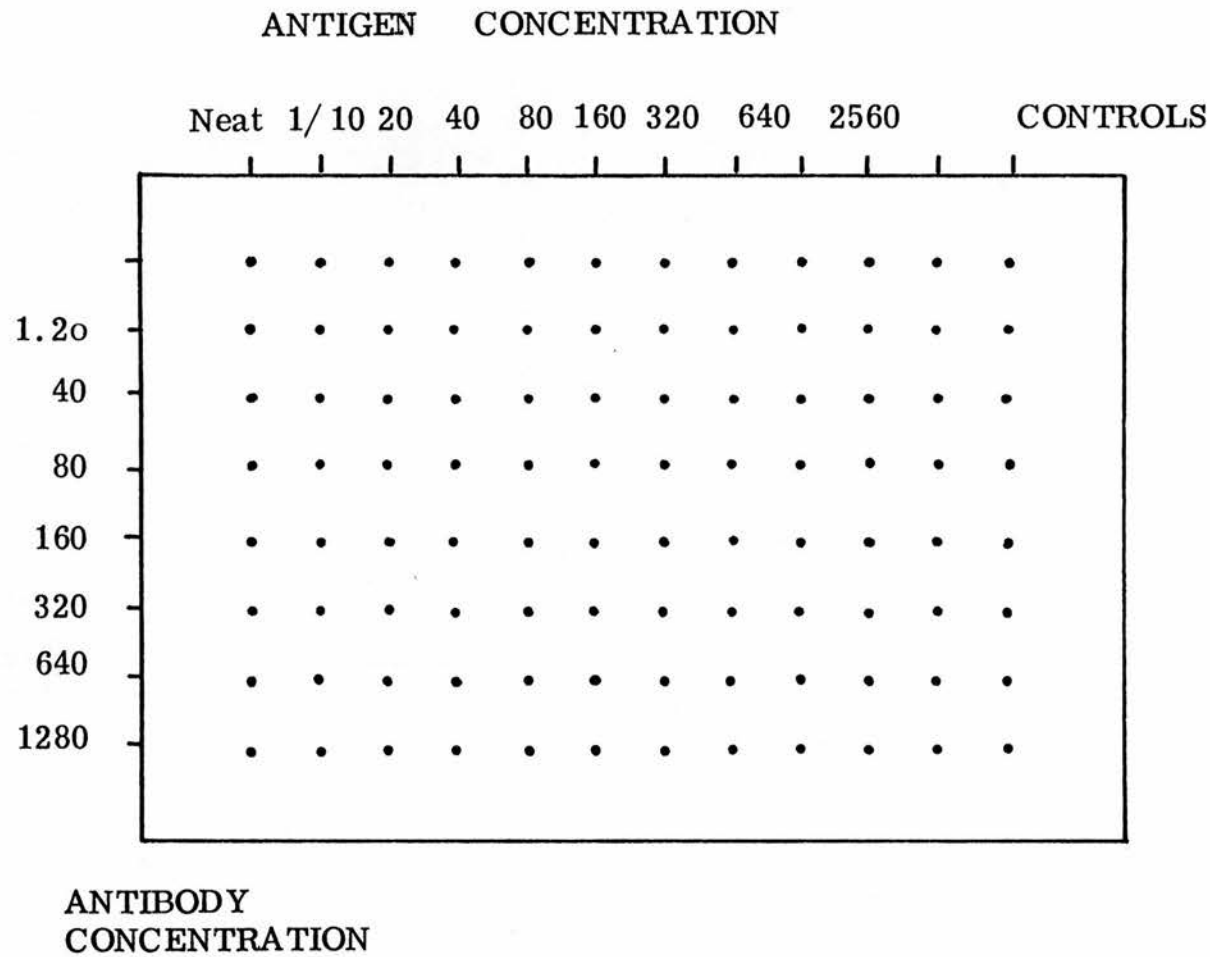
At the beginning of this investigation to adapt the enzyme-linked immunosorbent assay (ELISA) for grouping of streptococci, the standard 'indirect' ELISA technique of Poxton (1979), and described in the Methods section of this chapter was used.

Antigen extracts (0.4 ml) prepared by Lancefield's method, from the prototype strains representing the different serological streptococcal groups (A, B, C, D, G), were diluted in 4.5 ml of 0.05M carbonate buffer, pH 9.6. Doubling dilutions of these stock solutions were then made up and 50µl volumes were dispensed into the wells of microtitration plates. A separate plate was used for each of the group-specific antigens.

To give an indication of optimum concentrations of antigen and antisera in the tests, a checkerboard arrangement of doubling dilutions of the homologous antiserum were added (50µl) to wells of each microtitration plate (see Fig. 4.3). A standard dilution of conjugate in phosphate buffered saline, pH 7.4 (PBS) was used for all the initial tests, and similarly, the alkaline phosphatase substrate was maintained at a concentration of 1 mg/ml in carbonate buffer, pH 9.8 for all tests.

Incubation times for each of the stages were as described in the Methods section of this chapter.

Fig. 4.3 Assessment of optimum antigen/antibody concentrations for use in ELISA.



Theoretically, because homologous antiserum was added to wells containing specific antigen, it was expected that all wells would appear yellow due to degradation of the substrate solution by alkaline phosphatase. However, on addition of substrate to the test wells, results were haphazard and far from positive. Some of the wells showed a slight yellow colour but the majority remained colourless, denoting an absence of alkaline phosphatase conjugate. Repeated tests with the same method resulted in similar findings.

Three possible reasons for the failure of the tests were considered. First, the antigen preparation had not adhered to the polystyrene surfaces and was removed during the first washing procedure. This was thought most likely. Second, the antigen had adhered, but in combination with antiserum had been washed away, and third, the antigen-antibody-conjugate complex may have been removed during the final washing procedure before substrate was added.

A number of experiments were carried out in an attempt to improve the binding capability of the carbohydrate group-specific antigen to polystyrene surfaces. The pH of the diluent was altered for tests at increased acidity and alkalinity, PBS was tried as an alternative buffer system, coating was attempted with antigen in weak HCl (0.067M), antigen was diluted in normal saline only and placed in wells, and finally, incubation temperatures for binding were changed from $4^{o}C$ to $37^{o}C$ and $60^{o}C$. None of these variations brought about any increase in antigen binding, as judged by the ELISA test.

The next group of experiments concentrated on trying to alter the polystyrene surfaces of the microtitration plates. The plates were dipped briefly into a weak acid (0.067M HCl) and also into weak alkali (0.5M NaOH) to remove any inhibitory surface-active layers within the polystyrene. This treatment brought no improvement. The surfaces of the floors of the wells in the plates were roughened by scratching with a metal spatula, but this had no effect on antigen binding. The wells were coated with poly-L-lysine, which has been used previously for coating mouse lymphocytes to glass plates (Dr W.H. McBride, Department of Bacteriology, Edinburgh, personal communication). A solution of 50µg/ml poly-L-lysine in water was prepared and 50µl volumes were dispensed into wells of the microtitration plate. This was allowed to stand for four hours at room temperature and the plate was shaken dry. The standard ELISA technique was then performed with the prepared plate using the 'Lancefield' antigen in sodium carbonate buffer, pH 9.6. There was no significant difference in results obtained in tests with the prepared plate compared with the untreated plate.

At this time, I learned of the work being carried out by Dr B.M. Gray at the Department of Paediatrics and Microbiology, University of Alabama in Birmingham, U.S.A.. Dr Gray had also studied the binding capabilities of streptococcal carbohydrate antigens to polystyrene surfaces and had concluded that for unknown reasons, these specific streptococcal antigens were incapable of adhering firmly to polystyrene.

Accordingly I decided to adopt an alternative approach and investigate the possibility of binding whole, untreated

streptococcal cells to the polystyrene plates. Initially, only group B streptococci were used and cells from an overnight growth in 5 ml of Todd-Hewitt broth were collected and suspended in 5 ml of sodium carbonate buffer, pH 9.6. Volumes (50µl) were dispensed into each well and the plate was left open and incubated overnight at room temperature. Wells were washed three times with PBS-Tween and the plate was shaken dry. The ELISA procedure previously described was performed with the homologous antisera at a dilution of 1 in 400 in saline. The results recorded from this test were all strongly positive within 15 min of addition of substrate.

This technique was then applied with streptococcal cells of the prototype strains of groups A, C, D and G. In each case, strongly positive results in the ELISA were obtained with the respective homologous antisera. A checkerboard arrangement of doubling dilutions of antigen (cells from 5 ml of Todd-Hewitt broth in buffer) and antisera was set up as before (Fig. 4.3) to define optimum reaction concentrations. The results from this test indicated that 50µl of a 1000-fold dilution of antiserum in saline added to wells containing bound cells produced strongly positive results. Volumes (50µl) of cells in 5 ml of sodium carbonate buffer, pH 9.6, without further dilution, were the most convenient.

The next experiments were designed to assess the relative specificity of each of the grouping antisera reacted against cells of the prototype strains of A, B, C, D and G streptococci in the ELISA technique. Judged by the intensity of yellowness

produced by degradation of alkaline phosphatase substrate in the wells, the antisera diluted 1 in 1000 were completely non-specific, and differentiation of streptococci could not be achieved.

Absorption of cross-reacting antibodies from the different antisera with each of the prototype strains was performed by the method described earlier in this chapter. To obtain highly specific antisera which did not react with heterologous antigens for use in ELISA required between five to nine separate absorptions for each serum. In particular, group A antiserum exhibited marked cross-reactivity with group G cells, and similarly, group B serum with group G cells. When tested in the ELISA procedure, the absorbed sera were specific, but when used at the same dilution as previously, a decrease in activity was noted.

The final variable to be investigated in the adaptation of the ELISA system for grouping of beta-haemolytic streptococci, was the incubation times for each of the separate stages of ELISA. The method of Poxton (1979) originally used in this study required two days for completion of ELISA. In order to adapt the technique as a rapid alternative for grouping streptococci, incubation times for each of the separate stages were reduced to 1h. This diminution in time was achieved by incubating cells in the binding stage for 1h at 60^{o}C. All other incubations were performed at 37^{o}C.

The overall reduction in time for completion of ELISA, however, was at the expense of high serum concentrations, and it became necessary to use serum at a dilution of 1 in 400 in saline.

As a result of the various trials undertaken the following protocol was developed for rapid grouping of beta-haemolytic streptococci.

Streptococci were grown in 5 ml of Todd-Hewitt broth overnight at 37°C. The cells were washed once in saline and suspended in 0.5 ml of 0.05M sodium carbonate buffer at pH 9.6, containing 0.02% (w/v) of sodium azide. Volumes (50µl) were dispensed into wells of a microtitration plate and incubated in a drying cabinet at 60°C for 1h to allow cells to adhere to the polystyrene surface. Wells were washed three times with PBS-Tween and the plate was shaken dry. Volumes (50µl) of a 1 in 400 dilution of antiserum in PBS-Tween were dispensed into the wells and incubated at 37°C for 1h. To prevent evaporation, the plate was sealed in aluminium foil. Washing with PBS-Tween was repeated three times and the plate was shaken dry. Anti-rabbit antibody conjugated to alkaline phosphatase at a dilution of 1 in 500 in PBS-Tween was then dispensed into each well (50µl) and incubated for 1h at 37°C. Wells were again washed three times with PBS-Tween, shaken dry, and 50µl of substrate solution (1 mg of p-nitrophenylphosphate in 1 ml of 0.05M carbonate buffer, pH 9.8) was added. The plate was then left uncovered at room temperature. Results were read visually within 45 min of addition of substrate.

Section 4 - Streptococcal grouping by modified ELISA

One hundred streptococcal strains previously grouped by precipitation tests in agar gel (Section 2 of Results) were serologically identified by the modified ELISA technique. Complete agreement in the results was achieved with the two methods (Fig.4.4).

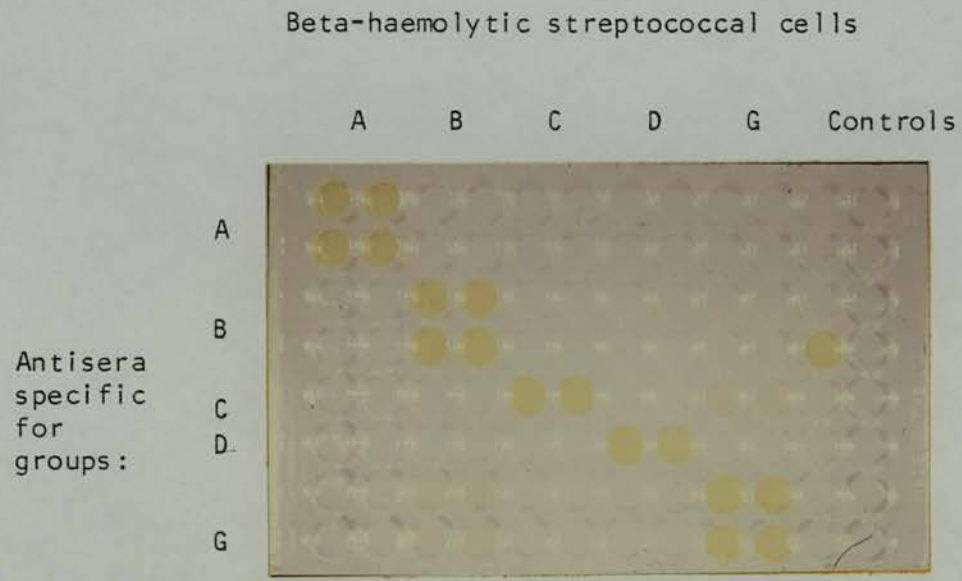


Fig. 4.4 ELISA technique for grouping beta-haemolytic streptococci.

(This plate shows reactions between Groups A, B, C, D and G and specific antisera).

Section 5 - 'Double-sandwich' ELISA technique

An attempt was made to adapt the ELISA technique for serological typing of group B streptococci. The difficulties already encountered in binding group-specific carbohydrate streptococcal antigens to polystyrene surfaces indicated that the 'double-sandwich' ELISA might be more successfully applied to this investigation. The double-sandwich ELISA technique was performed essentially as described in the Methods section of this chapter.

Preliminary experiments to assess the potency of the specific Ia immunoglobulin-alkaline phosphatase conjugate showed that in homologous Ia serotype reactions a conjugate titre of only 1 in 10 in PBS-Tween produced strongly positive results. This was regardless of various antigen-antiserum dilutions. Further experiments with this system to distinguish serotypes of group B streptococci were unsuccessful and this was attributed to lack of specificity of the Ia Ig-alkaline phosphatase conjugate. It was decided to discontinue this line of study. Additional comment will be presented in the Discussion section of this chapter.

Section 6 - Streptococcal grouping by commercial coagglutination tests

Beta-haemolytic streptococci previously identified by double-diffusion in agar gel and by the ELISA technique were distinguished by a commercially available coagglutination technique (Streptosec). Group D streptococcal strains were omitted from

the study since the kit was able to detect groups A, B, C and G only.

The coagglutination technique correctly identified 70 of the 72 strains tested (Fig. 4.5). Two strains of group A streptococci did not produce coagglutination with any of the grouping reagents.

Table 4.1 compares the times taken for coagglutination reactions to occur with strains incubated for 4h in Todd-Hewitt broth, and with the same strains picked directly from a blood agar plate. Since there was a limited supply of kits, only 51 strains were tested by the direct method. Over 90% of bacteria grown in Todd-Hewitt broth were positive within 60 sec, whereas only around 50% of strains tested directly from the blood agar plate reacted as quickly. All strains whose reactions took longer than 60 sec gave a positive reaction within 5 min.

The intensity of the coagglutination reactions is shown in Table 4.2. The strongest reactions were observed with those strains incubated in Todd-Hewitt broth. Reactions with those plated directly were mostly of moderate intensity, although a substantial proportion (24%) produced only a weak reaction.

There were no significant intergroup differences either in reaction times or in strength of coagglutination reactions. There was, however, a difference in cross-reactions observed between the streptococcal groups. Of the strains correctly identified, two group A organisms grown in Todd-Hewitt broth produced cross-reactions with the heterologous antibodies bound to staphylococcal cells. Nine strains (five of group A, four of group C) gave multiple coagglutinations when tested directly from the primary plate.

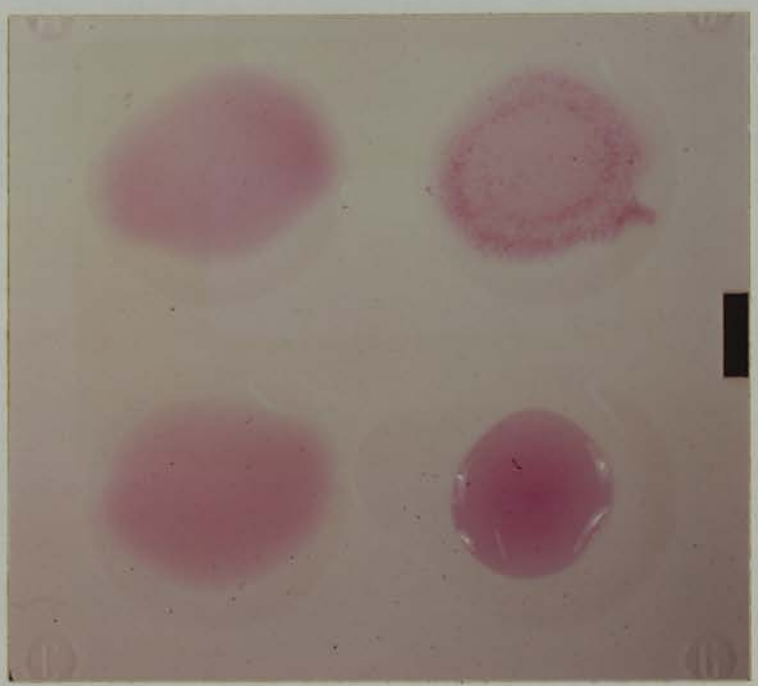


Fig. 4.5 Streptosec coagglutination test.

(This shows a positive reaction with the group B streptococcal reagents).

Table 4.1 Coagglutination reaction times of strains of beta-haemolytic streptococci.

	Number and percentage of strains reacting			
	THB incubation		Direct plating	
	Number	%	Number	%
Under 20 sec	48	66.6	17	33.3
Under 60 sec	66	94.3	25	49.0
Over 60 sec	4	5.7	26	51.0

Table 4.2 Strength of coagglutination reactions of strains of beta-haemolytic streptococci.

	Number and percentage of strains reacting			
	THB incubation		Direct plating	
	Number	%	Number	%
Strong	33	47.1	7	13.7
Moderate	36	51.5	32	62.8
Weak	1	1.4	12	23.5

Discussion

Serological grouping of beta-haemolytic streptococci in the diagnostic bacteriology laboratory is usually performed by one of two methods. The 'Lancefield' antigen extraction procedure followed by double diffusion against specific antisera in agar gel has been shown to be reliable but very time consuming. In addition, if antigen extraction is not performed carefully, especially at the neutralisation stage, false-positive and false-negative results may occur. In the present study, all 100 streptococcal strains tested were identified by this method, and little difficulty was experienced with heterologous cross-reactions.

The coagglutination technique originally described by Christensen et al. (1973) and now available in various commercial forms, has largely superseded the conventional 'Lancefield' extraction technique, and is widely used in Europe and the U.S.A.. The main advantage of this technique is that complicated antigen extraction procedures are unnecessary with consequent savings in time and labour. The new commercial preparation tested in this study was shown to be reliable and extremely rapid, but has the drawback of being very expensive. Cross-reactivity of strains grown in Todd-Hewitt broth and tested with heterologous antisera was not a significant hazard in identification of streptococcal groups. The same strains picked directly from the plate, however, did produce minor coagglutination reactions with the other grouping reagents. The manufacturer of this particular kit (Streptosec) recommends that if coagglutination occurs with more than one reagent, the greater or more rapid degree of coagglutination

should be taken as indicating the correct streptococcal group. If equal degrees of reaction occur the streptococcal suspension should be trypsinised and retested.

The enzyme-linked immunosorbent assay (ELISA) has been adapted extensively to measure antibody titres in serum against a variety of infectious agents, but has not been previously described as a technique for rapid serological groupjng of streptococci. Preliminary tests with the system, described in the Results section of this chapter, indicated that streptococcal carbohydrate antigens are unable to bind firmly to the polystyrene surfaces of a microtitration plate. Gray (1979) suggested that the main reason for poor binding to polystyrene is that the overall negative charge carried by the carbohydrates interferes with the adsorption mechanism. I cannot agree with this supposition. The fact that polystyrene by itself is an uncharged polymer, suggests that the relative charge of molecules in close contact to the surface has little, if any, inhibitory effect on binding capability. During the manufacture of polystyrene microtitration plates, the moulding templates are coated with compounds that facilitate removal of the completed plates from the templates. It may be that incorporation of these compounds into the surface layers of the polystyrene has in some way an inhibitory effect on binding of polysaccharide complexes.

The method outlined by Gray (1979) to overcome these problems of binding streptococcal carbohydrate antigens to a carrier surface is to couple the antigen to poly-lysine, with cyanuric chloride as the coupling agent. The poly-lysine is then adsorbed to the surfaces of plastic and polystyrene tubes. The procedure is

time-consuming and relatively complex, and has the main disadvantage of adding yet another stage in the grouping procedure for streptococci.

The indirect ELISA method for grouping beta-haemolytic streptococci outlined in this chapter was shown to be completely reliable and very rapid. Untreated, whole bacteria are used in the assay, and therefore the need for any extraction procedures is obviated. Results are read visually as being either definitely positive or negative, and spectrophotometric apparatus is unnecessary. Cross-reactivity between streptococcal groups is insignificant with highly absorbed antisera, and because the reagents used are of low concentrations in buffer solutions the test was found to be relatively inexpensive in comparison with the coagglutination technique.

The fact that there is a degree of complexity in the initial standardisation of the assay may make the ELISA system unattractive to laboratories dealing with only a few streptococcal samples daily. The role of ELISA for grouping purposes may, therefore, be limited to laboratories handling large numbers of specimens, and reference laboratories where quality control of antisera may not be so difficult.

During the preparation of this thesis, a report by Rote et al. (1980) at the University of Utah, Salt Lake City, U.S.A., described an ELISA system to distinguish group B streptococcal antigens. I was astonished to learn that the method used was almost exactly the same as that described in this chapter. Whole cells were used as antigen preparations, incubation times

for each of the stages were 1h at 37°C, and finally, the antisera and conjugate dilutions were similar to that described in this chapter. The authors also used the same method for measuring antibody levels against group B organisms in patients' serum.

The enzyme-linked immunosorbent assay was not successfully adapted for serological typing of group B streptococci. Typing antisera obtained from rabbits by intravenous injection of whole cell preparations of the serotypes of group B were shown later (Chapter 5) to contain a range of cross-reactive antibodies. It was found impossible to absorb out these antibodies, while at the same time maintain a degree of intrinsic reactivity within the respective antisera. It is likely that the only method to obtain truly type-specific antisera for group B streptococci is by raising monospecific antibodies following the development of hybrid myeloma cell lines. Further investigations to elucidate the confusing range of group B streptococcal antigens present in the serotypes will also be helpful to obtain a clearer understanding of the immunological profile of these bacteria.

Chapter 5

Immunochemical studies on the antigens of group B streptococci

Introduction

Classification of group B streptococci

Hitchcock (1924) first demonstrated that most streptococci from human infections possess a serologically active polysaccharide antigen. In 1928 and 1933, Lancefield reported that beta-haemolytic streptococci from various sources could be differentiated into serological groups. These 'Lancefield groups' corresponded in general with those groups described by earlier investigators on the basis of cultural and biochemical characteristics. The method employed by Lancefield to achieve serological grouping of streptococci involved the extraction of a carbohydrate antigen, called the "C substance", from the cell walls of the organisms by hot hydrochloric acid, and reacting the extract with specific antisera raised in rabbits against whole streptococcal cell preparations. This technique enabled Lancefield to recognise seven serological groups, labelled A to G, and *Streptococcus agalactiae* was designated as group B. Since these studies by Lancefield, streptococcal taxonomy has been expanded to accommodate 13 additional serological groups labelled H to V, omitting I and J (Wilkinson, 1978).

Stableforth (1932) examined the serological characteristics of streptococcal strains causing bovine mastitis, namely '*Streptococcus mastidis*' (*Strept. agalactiae*), and labelled the strains as group I (Lancefield's group B). Further serological investigations (1937, 1946) by Stableforth on his group I strains, initially using Lancefield's precipitation technique (1933), and

later by microscopic slide agglutination, lead to the recognition of 16 definite types: 1a, 1b, 1c, 1d, 1e, 1f, 2a, 3a, 3b, 3c, 3d, 3e, 4a, 4b, 6a, 7a.

In 1934, Lancefield subdivided group B streptococci (GBS) into three serologically distinct types: I, II and III, by means of precipitation tests with hot-acid extracts. She confirmed this by passive protection tests in mice, and identified the type-specific antigens as polysaccharides and called them "S substances". In 1938 Lancefield reported two distinct sub-types in her type I and called them Ia and Ib.

During the late 1930's considerable confusion was caused by the existence of the two serological typing systems of Lancefield and Stableforth in the classification of GBS. The International Congress for Microbiology in 1936 suggested that in order to avoid further confusion, Lancefield's method of classification of streptococcal groups and types should be adopted. At this time, the fact that Stableforth had reported 16 definite GBS types and Lancefield had recognised only four distinct serological types was left unexplained.

In 1955, however, Pattison et al. compared distinct serological types of Lancefield and Stableforth, and found that most of the Stableforth types could be incorporated within the four Lancefield types.

Pattison et al. (1955b) isolated GBS strains of bovine origin which lacked a polysaccharide type antigen, but possessed protein antigens that could be extracted with hot acid and detected in precipitation tests. They designated these antigens X and Y.

The Y protein was trypsin resistant, but pepsin sensitive, and indistinguishable from the R antigen of group A streptococcus type 28 strain (Wilkinson, 1972). The X protein was sensitive to both trypsin and pepsin. A number of workers (Pattison et al., 1955b; Butter and de Moor, 1967; Jelinkova et al., 1970) have reported that most bovine GBS strains have protein type-antigens, but many are devoid of polysaccharide type antigens, whereas human strains nearly always have a polysaccharide type-antigen and few have protein type-antigens.

Wilkinson and Eagon (1971) proposed yet another sub-type within the type 1 series of organisms. This was called the Ic serotype and possessed antigens in common with types Ia and Ib, but no new antigen specific for Ic was demonstrated. Type Ic strains possess the Ia polysaccharide antigen and an additional protein antigen Ibc, which is also present, along with the polysaccharide Ib, in members of the Ib serotype. Thus the antigenic constitution of the three subtypes of type I are:

type Ia - Ia polysaccharide; type Ib - Ib polysaccharide and Ibc protein; type Ic - Ia polysaccharide and Ibc protein (Wilkinson, 1975; Lancefield et al., 1975). All members of serotype I have, in addition to the previously described antigens, a common polysaccharide antigen Iabc which is also cross-protective in mice (Lancefield et al., 1975).

Recently Kjems and Henrichsen (1979) have raised antiserum to a previously unrecognised serotype of GBS. They suggested that the new antigen was probably polysaccharide in nature and that strains containing this antigen should be designated type IV.

Chemical composition of GBS cell wall (group-specific) antigens

Cummins and Harris (1956) extensively reviewed the chemical composition of cell walls in many Gram-positive bacteria. They described the cell wall as being "very tough and extremely insoluble in a wide variety of solvents". The main components in the wall were sugars and amino acids and were therefore called 'mucosubstances'. Walls from one strain of GBS (NCTC 6175) were examined and found to contain rhamnose, galactose, glucosamine, and an unknown hexosamine, as well as the amino acids alanine, glutamic acid and lysine. No attempts were made in this study to quantify the components relative to each other. Barkulis and Jones (1957) were among the first to investigate the chemistry of streptococcal cell walls. In their study, walls were isolated from group A streptococci after cell breakage in a Mickle disintegrator and washed in water to remove nucleic acid components. The walls were found to constitute approximately 25% of the dry weight of cells and were composed of D-rhamnose and hexosamine (33%) which made up the 'C substance', and unidentified protein (67%).

By 1961, it was realised that the cell walls of most Gram-positive bacteria were broadly similar and that the basic structural component responsible for maintaining rigidity to the cell was the cell wall mucopeptide, now known as peptidoglycan (Krause and McCarty, 1961). This component was shown to be composed of N-acetylglucosamine, N-acetylmuramic acid, and a limited number of amino acids, in the case of group A streptococci these were alanine, lysine, glycine and glutamic acid. Furthermore, Krause and McCarty suggested that the mucopeptide component, although associated with, was chemically distinct from the other cell wall component in

streptococci, the group-specific antigen or 'C substance'.

Slade and Slamp (1962) reported on the composition of cell walls of bacteria from 17 serological groups of streptococci, including group B. Rhamnose was the major constituent of the carbohydrate moiety in bacteria of all the groups except groups K and R which contained none. In addition to rhamnose, GBS cell wall carbohydrate contained glucose and galactose in equal amounts. It was thought that since the chemical composition of the C carbohydrate of many of the serological groups was so similar that specificity of each group relied upon a unique stereochemical arrangement of the sugars within the cell wall.

Wittner and Hayashi (1965) concentrated on GBS cell walls only. Carbohydrate composition of cell walls of GBS serotypes Ia, Ib, II and III were analysed and found to contain almost identical proportions of sugars, namely rhamnose (10%), hexoses (18%) which were mainly galactose and a small amount of glucose, and hexosamine (9%) which was mainly glucosamine and a trace of galactosamine. The above workers also separated carbohydrates from the walls of serotype II into two portions by ethanol fractionation, when serologically tested one portion showed group activity and was rich in rhamnose, and the other portion showed type-specific serological activity and contained mainly galactose and no rhamnose. The importance of rhamnose as an antigenic determinant in the group-specific carbohydrate of GBS was also noted by Curtis and Krause (1964a, 1964b). They found that the group B antigen was composed of 50% rhamnose, 12% glucosamine and 15% galactose. Sugar inhibition tests revealed that at a final concentration of 1%, rhamnose markedly inhibited the group B

precipitin reaction, whereas the other constituent sugars had no appreciable effect. A chance finding indicated that rhamnose also inhibited the group G precipitin reaction, suggesting that it occupied a terminal position in the antigenic determinants of both B and G carbohydrates. It was suggested that this might explain the reason for cross-reactivity in precipitation tests involving groups B and G.

Most investigations relating to streptococcal cell walls have examined the chemical and immunological profiles of the secondary wall polymer, the group-specific carbohydrate antigen. Recently, however, attempts have been made to assess antigenicity of the peptidoglycan (mucopeptide) component of cell walls. Following the earlier description of the constituents of this moiety (Krause and McCarty, 1961), Karakawa and Krause (1966) redefined peptidoglycan as being composed of three principle chemical complexes; the polymer of N-acetylglucosamine and N-acetylmuramic acid, a peptide linked to the muramic acid residue of the hexosamine polymer, and the peptide bridge forming cross-linkages between the peptides of adjacent polymers (see Fig. 5.1 and Fig. 5.2). In view of the complexity of this structure it was thought that the immune response to peptidoglycan might be directed against several of its chemical components. Karakawa et al. (1967) reported that immunisation of rabbits with streptococcal vaccines (groups A, C and D) gave rise to sets of antibodies with specificity for both the hexosamine polymer of cell wall peptidoglycan and also against the peptide moiety linked to muramic acid.

The true biological significance of streptococcal peptidoglycan

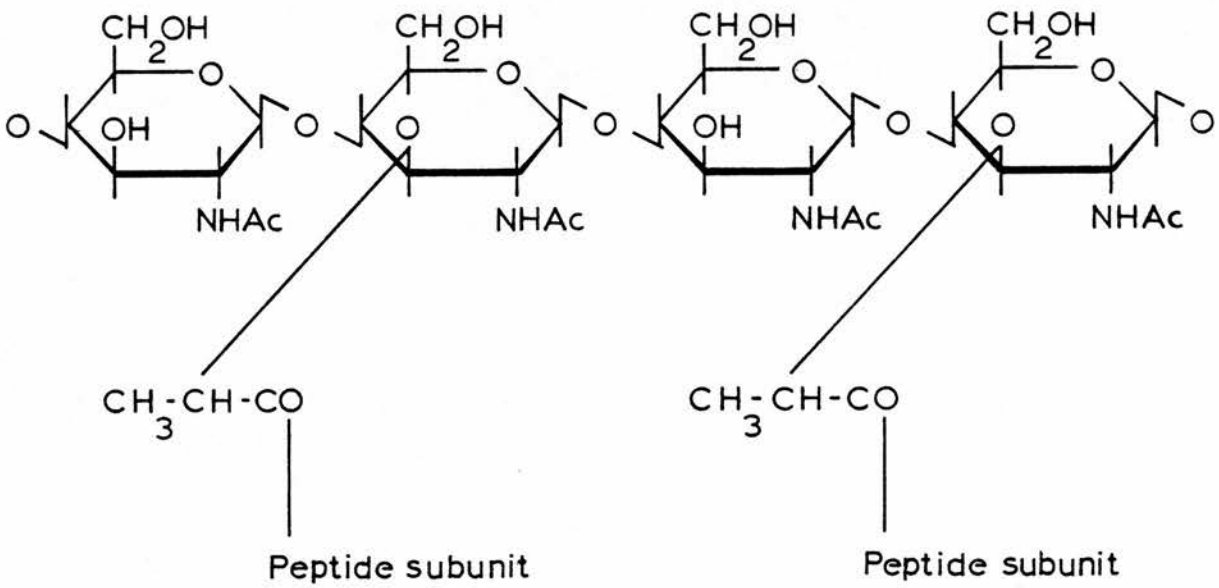
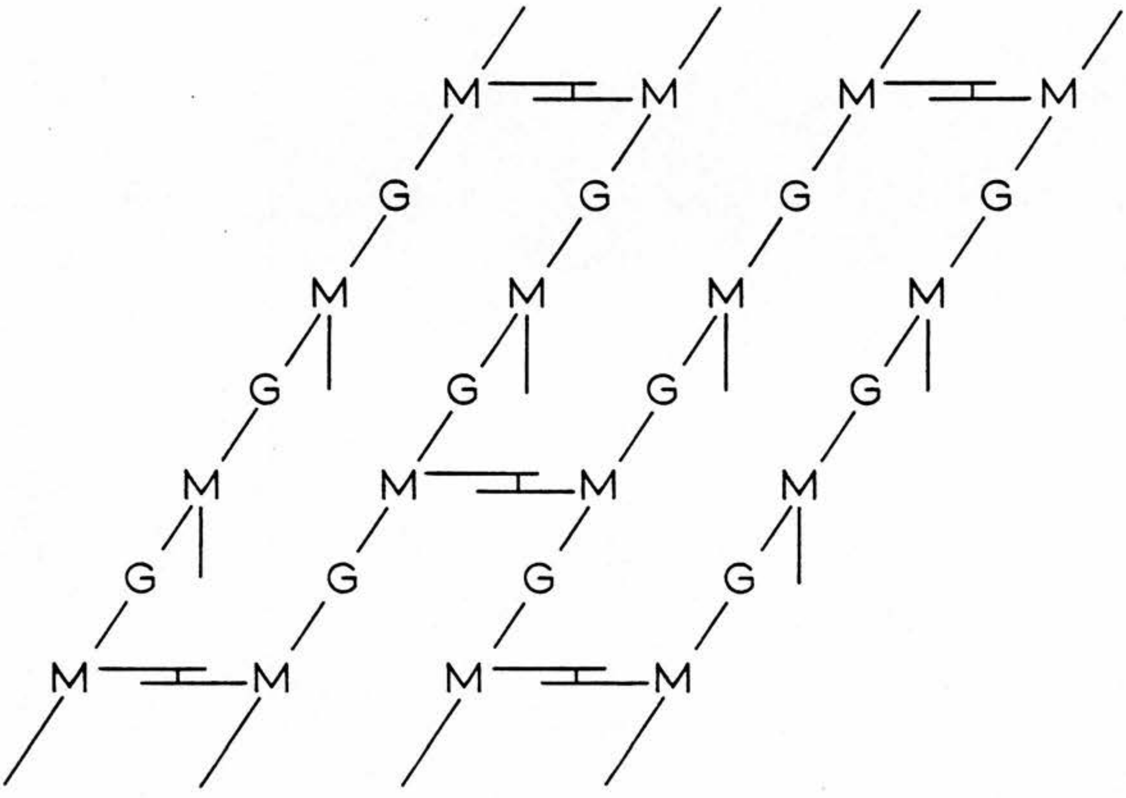


Fig. 5.1 Structure of the peptidoglycan backbone in Gram-positive bacteria (From Ghuysen, 1968).



G=N-Ac Glucosamine

M=N-Ac Muramic acid

Fig. 5.2 Monolayer of peptidoglycan of Gram-positive bacteria (From Ghuysen, 1968).

has yet to be clarified. Under appropriate conditions, peptidoglycan has some of the biological properties of endotoxin. Rotta et al. (1965) showed that streptococcal cell wall peptidoglycan induced fever in rabbits and enhanced non-specific resistance in mice against subsequent challenge with streptococci. Abdulla and Schwab (1966) observed dermal necrosis following injection of peptidoglycan into the skin of rabbits. It was thought possible that these properties were mainly dependent on the inherent toxocity of peptidoglycan and had little to do with antigenicity. Stetson (1965), however, argued that endotoxins possessed biological activity only by virtue of their antigenicity and that the toxic reactions produced by peptidoglycan may be manifestations of acquired immunity.

Chemical composition of GBS surface (type-specific) antigens

The type-specific antigens of GBS consist of surface or capsular polysaccharides and proteins which are chemically and serologically distinct from the group-specific antigen contained within the cell wall (Lancefield and Freimer, 1966). These workers employed two methods to extract surface antigens from GBS type II organisms. Hydrolysis with hot hydrochloric acid resulted in the detection of an antigen, composed of galactose, glucose and glucosamine, which reacted specifically with type II antisera. The same three sugars were released by hydrolysis with cold trichloroacetic acid (TCA) as well as an additional serologically active component which was not identified. This component was destroyed by heating the TCA-extracted antigen with dilute HCl, leaving a chemically and immunologically identical

antigen as the HCl extract. Freimer (1967) reviewed the findings of his earlier report and added that great difficulty was being encountered in identifying the unknown type II determinant.

Wilkinson and Eagon (1971) reported that the type-specific polysaccharide antigens shared by serotypes Ia and Ic contained 69% galactose and 31% N-acetylglucosamine. The antigens were extracted from whole cells by hydrolysis with hot HCl of low molarity. Sugar inhibition tests with the hydrolysed monosaccharides, however, failed to significantly inhibit the precipitin reaction between the antigens and homologous antisera, indicating that an important determinant group on the antigen had been lost during the extraction procedure. Russell and Norcross (1972) described the chemical components of the type III antigen. The predominant constituents were galactose, glucose and glucosamine in a ratio of 2:1:1 respectively. Muramic acid, and the amino acids, glutamic acid, serine, alanine, and aspartic acid were present in minute quantities and it was proposed that these complexes were contaminants non-covalently linked to the polysaccharide antigen. Of great interest was the detection of glucuronic acid in the extract which amounted to 3.1% of the total antigen content. Serological inhibition tests with glucuronic acid resulted in a 34% precipitin inhibition, suggesting that this substance was indeed the previously unrecognised antigenic determinant group of GBS type III strains.

In 1975, Wilkinson confirmed her earlier findings (Wilkinson and Eagon, 1971) in relation to chemical composition of serotypes Ia, Ib and Ic, but on this occasion she used a different technique of antigen extraction. This involved washing whole cells in a

saline solution only. In addition to the previously reported monosaccharides, this method also enabled the detection of sialic acid. The author attached little importance to this finding and suggested that the presence of sialic acid in the extract probably increased the net negative charge.

By 1976, it was realised that the classical methods of extracting polysaccharide type-specific antigens from GBS by hot HCl treatment of whole organisms resulted in partially degraded antigen complexes which were of low molecular weight and were immunologically incomplete (Baker et al., 1976). Isolation of the complete native antigen by more subtle extraction procedures, such as washing whole cells in buffered saline, enabled the isolation of antigen complexes which were theoretically more similar to those which elicited an immune response in the host. Using the saline-wash technique, Baker et al. (1976) isolated mannose and sialic acid in addition to the three monosaccharides previously described from the type-specific antigen of type III GBS. They suggested that the presence of sialic acid in the antigen was the predominant factor in overcoming host resistance in the pathogenesis of neonatal meningitis. Sialic acid in significant proportions in the GBS Ia serotype was confirmed by Kane and Karakawa (1977a) and later that year, these workers presented evidence to suggest that sialic acid was just one of two antigenic determinants present in the Ia antigen (Kane and Karakawa, 1977b). Rabbits immunised with GBS type Ia produced two distinct populations of protective antibodies. One of the antibody populations was shown to be active against terminal sialic acid

residues, whereas the other reacted with a galactosyl oligosaccharide determinant.

Tai et al. (1979) isolated from whole cells the Ib type-specific antigens, which contained galactose, glucose, N-acetylglucosamine and sialic acid in the approximate molar ratio of 2:1:1:1. The fact that this antigen cross-reacted with both type Ia and Ic antisera was attributed to the common determinant Iabc (Lancefield et al., 1975). Absorption studies indicated that the Ib specific determinant and the Iabc determinant were on the same molecule but that sialic acid was not the cross-reactive determinant (Tai et al., 1979). In 1980, Carey et al. described a method for the isolation of native GBS type-specific antigens from broth cultures. The chemical profile of the type III antigen was similar to that previously described.

Because the polysaccharide type-specific antigens were shown to be associated with the virulence of the respective organism type (Lancefield and Freimer, 1966), little attention has been directed towards the immunochemical properties of the protein antigens of GBS serotypes. It was first noted by Maxted (1949) that protein antigens of GBS were resistant to digestion by trypsin but not pepsin. Pattison et al. (1955b) showed two distinct protein antigens in GBS strains examined. One of these was serologically identical to the streptococcal group A 28R antigen, the other, which they designated X, was serologically distinct but cross-reactive with anti-R serum. Wilkinson and Eagon (1971) characterised the protein type antigen of GBS type Ic serologically and chemically. They found that it contained two determinants,

one susceptible to pepsin and trypsin digestion, and the other to pepsin but not trypsin. Both these determinants were also found in Ib strains. In addition, these authors showed that the protein antigen was serologically distinguishable from the two proteins R and X.

The cell wall and related structures of GBS is shown diagramatically in Fig. 5.3.

AIMS OF THE PRESENT STUDY

(1) To isolate and purify the cell wall carbohydrate antigens from GBS serotypes Ia, Ib and Ic, and to compare the results of different extraction techniques.

(2) To perform a chemical analysis of these antigens.

(3) To investigate the immunological role of the cell wall antigens, and to attempt to elucidate their relative functions in laboratory serological identification tests.

(4) To compare the immunological profile of the cell wall antigens with antigens extracted by the classical Lancefield hot HCl technique.

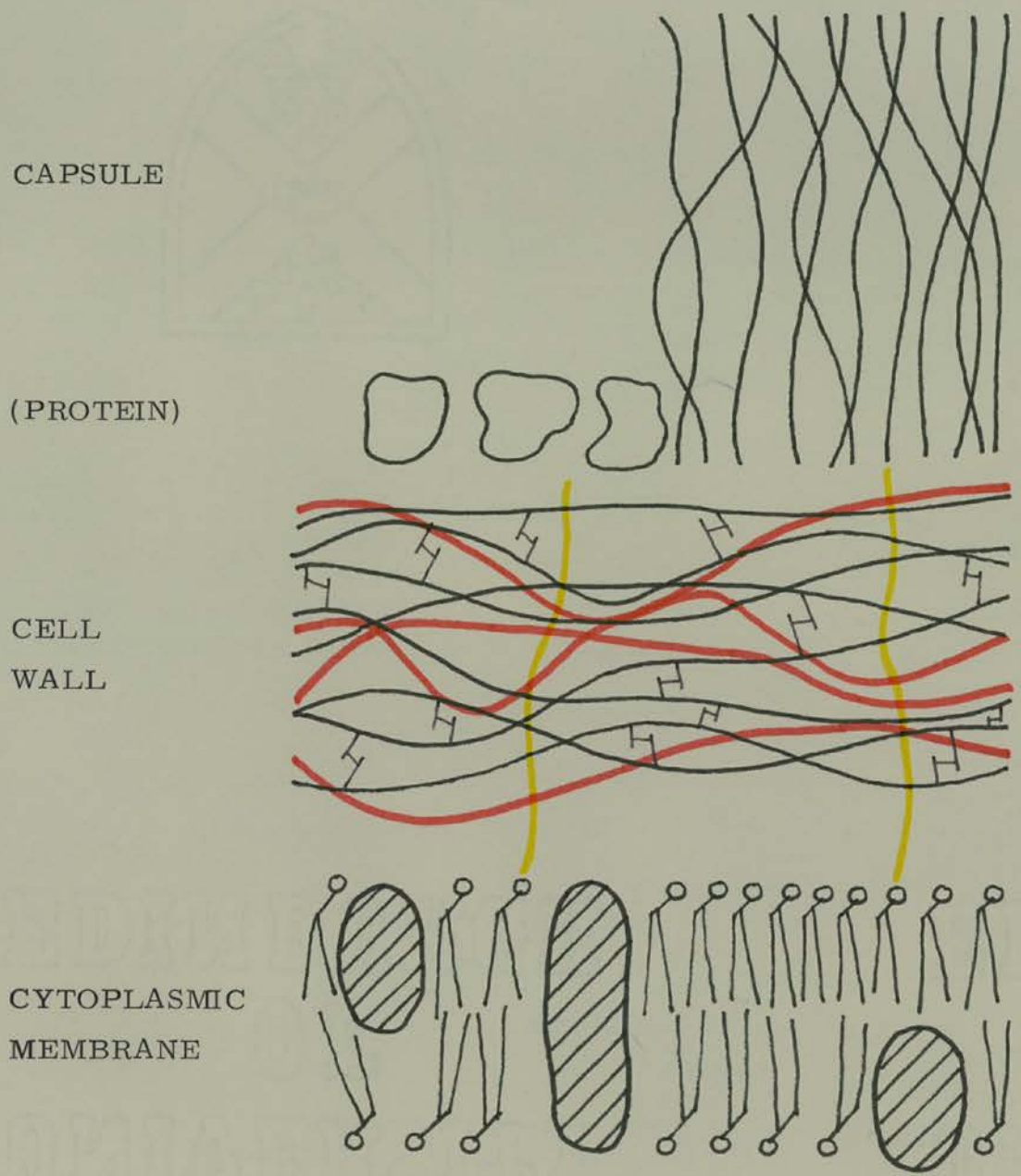


Fig. 5.3 Representation of the cell wall of group B beta-haemolytic streptococci.

Material

Bacteria

The following group B streptococcal strains representative of the main serotypes were obtained from the Streptococcal Reference Laboratory, Colindale, London: Ia (090R), Ib (H36B), Ic (NCTC 11078), II (NCTC 11079), III (NCTC 11080). Strains were maintained on nutrient agar slopes in screw-capped bottles at $4^{o}C$.

Growth media

Todd-Hewitt broth (Oxoid Ltd, London, U.K.) was routinely used for all liquid cultures, and solid media was 5-10% human blood agar plates.

Antisera

Preparation of both group and type-specific antisera for GBS was described earlier in this thesis (Chapter 4).

Crossed immunoelectrophoresis (CIE) reagents

Buffer

A barbital glycine Tris buffer described by Weeke (1973a) was prepared as follows:

Solution 1 26.0g of barbital-Na
4.14g of barbital
dissolved in 2 litres of water

Solution 2 112.4g of glycine
90.4g of Tris
dissolved in 2 litres of water

The final CIE buffer was obtained by mixing together equal volumes of solutions 1 and 2.

Agar gel

Agar was prepared as follows:

125 ml of barbital/glycine Tris buffer

375 ml of water

5g of agarose (BDH Electrophoresis grade)

The constituents were boiled for a few minutes and 5 ml Triton X100 (BDH Chemicals Ltd, Poole, U.K.) was added. Agar was dispensed into 15 ml amounts and stored at $4^{o}C$ until required.

Methods

Preparation of purified cell walls

Cell walls were prepared from bacteria of GBS serotypes Ia, Ib and Ic by the following methods.

Bacteria were cultured in 6 litres of Todd-Hewitt broth overnight at $37^{o}C$. Cells were harvested and washed sequentially in three volumes (50 ml) of saline (0.85% w/v NaCl) by centrifugation at 1000g for 15 min. The cells were suspended in 40 ml of ice-cold water and disrupted by passing through a French press (Aminco Inc., Maryland, U.S.A.) at 7000 psi. Cell walls and unbroken bacteria were collected by centrifugation at 52000g for 15 min at $4^{o}C$. The upper layer of the pellet consisting of the cell walls was separated from the lower layer of unbroken bacteria by careful suspension of the walls into water. Unbroken bacteria were returned to the French press and disrupted until complete

cellular breakage was achieved. Degree of breakage was assessed by examination of wet films under the microscope.

The suspension of cell walls in 20 ml of water was heated at 80°C for 3 min to destroy autolytic enzymes, then washed in a fresh volume (20 ml) of water by centrifugation at 1000g for 15 min at 4°C. The wet cell walls were then weighed and a 30% (w/v) suspension of walls in water was prepared. Protein and cell membrane components were removed from the suspension by adding an equal volume of boiling 5% (w/v) sodium dodecyl sulphate (SDS, BDH Chemicals Ltd) in water to the cell suspension and stirring for 4h at room temperature. To remove SDS, the cell walls were washed by centrifugation at 50000g for 10 min in six successive changes of water (15 ml) at 20°C. The reason for maintaining 20°C throughout the washing process was that SDS crystallised at temperatures lower than this.

The purified cell walls were then freeze-dried overnight, carefully weighed, and separated into a number of portions which were stored in sealed tubes until use.

Carbohydrate extraction from cell walls

Carbohydrates were extracted from the purified cell walls of GBS types Ia, Ib and Ic by hydrolysis with trichloroacetic acid. For type Ia cell walls only, two additional extraction procedures with hydrochloric acid and sodium hydroxide were performed in order to assess the effect of different extraction conditions on final carbohydrate structure and composition.

(1) Trichloroacetic acid technique. Samples of freeze-dried cell walls (75 mg) were suspended in 10 ml of ice-cold water.

Carbohydrate was extracted from the walls using 10 ml of 10% (w/v) trichloroacetic acid (TCA) and stirring for 48h at 4°C. The walls were removed by centrifugation at 50000g for 10 min and the supernate containing carbohydrate was collected. TCA was removed from the supernatant fluid by six successive extractions with 10 ml volumes of diethyl ether. Water was removed by rotary evaporation and the extracted carbohydrate was dissolved in 1 ml of water.

(2) Hydrochloric acid technique. The second sample (50 mg) of GBS type Ia cell walls were suspended in 7 ml of 0.1M HCl and stirred for 4h at 4°C. Walls were removed by centrifugation at 50000g for 10 min and the supernatant fluid was collected. Neutralisation of the fluid was performed with 0.2M NaOH. Water was removed by rotary evaporation and the extracted carbohydrate was dissolved in 1 ml of water.

(3) Sodium hydroxide technique. This technique was performed on GBS type Ia cell walls according to the method described by Anderson and Archibald (1975). Cell walls (60 mg) were suspended in 10 ml of 0.5M NaOH and stirred at room temperature for 1.5h. Walls were again removed by centrifugation at 50000g for 10 min and the supernate was collected.

Alkali was removed from the supernate by ion exchange chromatography. A column containing 7 ml of Dowex 50 (Standard H^+ form, 50-100 mesh size) in 1M ammonium sulphate was prepared, and two column volumes of 1M $(NH_3)_2SO_4$ were washed through to ensure complete exchange of NH_4^+ ions. Excess ammonium salt was removed by washing the column with six volumes (10 ml) of water. The sample (10 ml) was then transferred to the column

and washed through with water. The eluate was rotary evaporated to dryness and the remaining carbohydrate was dissolved in 1 ml of water.

Estimation of carbohydrate content of carbohydrate samples

Carbohydrate concentration of each of the samples was estimated according to the phenol-sulphuric acid method of Dubois et al. (1956) using a prepared standard of 0.117 mg/ml of glucose in water. The carbohydrate concentration of the samples was adjusted to 1 mg/ml in water.

Purification and separation of cell-wall carbohydrates

Carbohydrates released from the cell-walls were purified by performing ion-exchange chromatography. Samples (5 ml) of carbohydrate (1 mg/ml) in water were applied to a column (15mm x 170mm) of DE-52 DEAE-cellulose previously treated with 1M pyridinium acetate, pH 5.3. After washing with water (200 ml) to remove neutral products the column was eluted by a linear gradient of 100 ml of pyridinium acetate, pH 5.3, from 0 to 1M concentration. Fifty fractions (2 ml) were collected and analysed for carbohydrate by the method of Dubois et al. (1956). Tubes found to contain purified carbohydrate were stored at $4^{o}C$ until further use.

Preparation of 'Lancefield' GBS group and type-specific antigens

This was performed by the hot hydrochloric acid extraction technique of Lancefield (1933, 1934) and described in Chapter 4. The antigens diluted in water were stored at $4^{o}C$ until use.

Crossed immunoelectrophoresis (CIE) technique

Electrophoresis was carried out using a Shandon Southern apparatus (Camberley, Surrey, U.K.). The techniques employed for CIE were based on those described by Weeke (1973b). Agar gels used throughout the series of experiments for both first and second dimension electrophoresis consisted of agarose (BDH electrophoresis grade) at a final concentration of 1% by weight in a barbital/glycine Tris buffer, pH 8.8 described by Weeke (1973a).

Gels for the first dimension of CIE were prepared by pouring 15 ml of agar onto the surface of a glass plate (10 cm^2). The plate was then placed onto a template (see Fig. 5.4) and wells were punched in the gel at prescribed intervals according to the design of the template. Samples of carbohydrate (10 µl containing 1 mg/ml carbohydrate) extracted from cell walls and from whole cells (Lancefield' antigens) were dispensed into the wells, and the gel-slide was transferred to the Shandon electrophoresis apparatus. The electrode troughs contained 250 ml of barbital/glycine Tris buffer and filter paper wicks extending from the buffer compartments were arranged to just touch the ends of the gel slide. Electrophoresis was carried out at a constant voltage (12.5 v/cm) for 2h at $4^{o}C$; the carbohydrates migrated towards the anode through the gel.

The gel-slide was removed from the Shandon apparatus and again superimposed onto the template. Strips of gel approximately 1cm wide into which carbohydrate had migrated were cut out and transferred onto smaller glass slides (5cm^2) ready for the second dimension of CIE. To the remaining part of the slide not covered by the agar strip, a mixture of 3 ml of agarose and 0.5 ml of

specific GBS antiserum at a temperature of 52°C was added (Fig. 5.5). The slide was then returned to the Shandon apparatus at 4°C, the filter paper wicks were arranged to maintain continuity of electrical current across the gel slide, and a constant voltage of 12 v/cm was set to run for 16h at 4°C. In this second phase of electrophoresis the carbohydrate separated in the first dimension was run from the cathode towards the anode into the agar containing antiserum.

On completion of the second dimension of electrophoresis the gel slide was first covered with a layer of filter paper (Whatman No. 1) and compressed under a few pounds pressure for 15 min. The slide was then immersed in three successive volumes (20 ml) of 0.1M NaCl and one volume (20 ml) of water for 15 min each to remove unprecipitated protein components of the antiserum present in the agar. Following a further 15 min period of pressing to remove water, the gel was finally dried using a converted hair dryer.

Immobilised precipitin lines formed by reaction of the carbohydrate antigen and specific GBS antisera in the gel were visualised by staining with Coomassie blue for at least 15 min (Weeke, 1973a). Destaining of the background agar was achieved using an ethanol/acetic acid/water (1:2.5:6.5) destaining solution.

Analysis for phosphate in carbohydrate samples

Each of the purified carbohydrate samples were analysed for phosphate by the method described by Chen (1956).

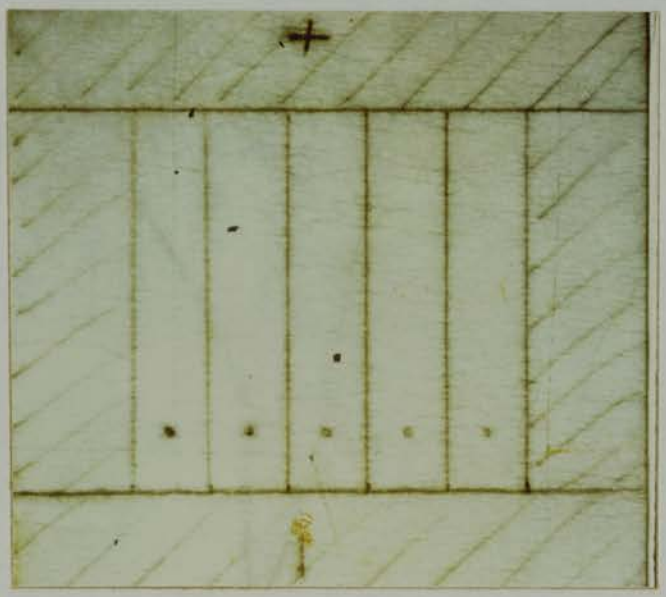


Fig. 5.4 Design of template for the first dimension of CIE. The position of the wells in the gel are denoted.

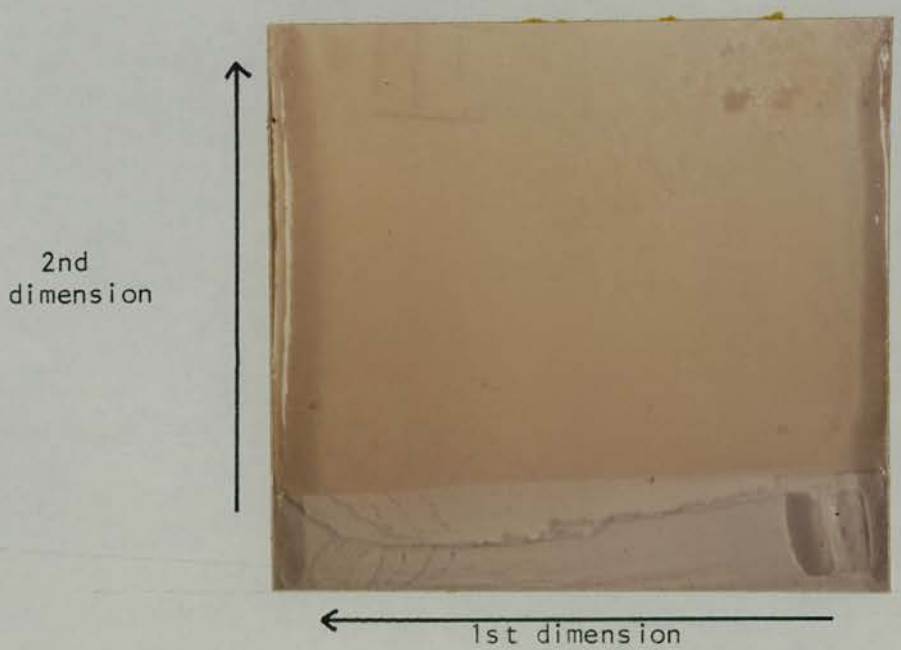


Fig. 5.5 Gel slide prepared for the second dimension of CIE. The coloured agar contains specific antiserum.

Acid hydrolysis of the cell-wall carbohydrate

Samples (1 ml) of the eluates from the DEAE-cellulose columns were hydrolysed in either 2M HCl for 4h at 100°C or 4M HCl for 18h at 100°C. Acid was removed by desiccation over NaOH and P_2O_5 *in vacuo* and the hydrolysates were dissolved in 1.5 ml of water.

Paper chromatography of carbohydrate hydrolysates

Descending paper chromatography was performed on Whatman No. 1 paper with a pyridine/acetic acid/water (6:4:3) solvent. Aminosugars were detected by ninhydrin spray (BDH Chemicals Ltd, Poole, U.K.) and reducing sugars by the alkaline silver nitrate reagents (Trevelyan *et al.*, 1950). Standards included in the tests were glucosamine and galactosamine and the reducing sugars were glucose, glucuronic acid, ribose, galactose, rhamnose and mannose.

Preparation of alditol acetates for gas-liquid chromatography

Hydrolysed samples were converted to alditol acetates by the following method. Hydrolysates (1 ml) were reduced by 10 mg of potassium borohydride for 1h at 20°C. Excess borohydride was destroyed by addition of three drops (0.06 ml) of glacial acetic acid. Methyl borate was removed from the samples by three successive distillations with methanol. The residue of each sample was then dissolved in 0.5 ml of pyridine and 0.5 ml of acetic anhydride and heated for 1h at 100°C in a sealed flask. Pyridine and excess acetic anhydride were removed by rotary

evaporation with successive volumes (5 ml) of toluene until the acetate residues were dry. Impurities in the residues were removed by adding 2 ml of chloroform and vortexing with 5 ml of water. The water was discarded and the chloroform phase was subjected to rotary evaporation. The resulting samples were redissolved in 0.5 ml of chloroform and analysed by gas-liquid chromatography.

Gas-liquid chromatography conditions

Alditol acetates were identified in a Pye Unicam Series 104 Chromatograph. A column (2.1m x 4mm) containing 3% OV-225 on 100-120 mesh Gas Chrom Q enabled identification of amino sugars; hexoses and pentoses were identified on a column (1.5m x 2mm) of 3% SP-2330 on 100-120 mesh Supelcoport. Gas flow rates for the OV-225 column were 35 ml/min of N_2, 35 ml/min of H_2, and 525 ml/min of air. Flow rates for SP-2330 were 20 ml/min of N_2, 20 ml/min of H_2, and 525 ml/min of air. The temperature of both columns was 180°C rising by 2°C/min to 240°C.

Determination of sialic acid concentrations

A series of experiments were performed which culminated in the determination of sialic acid concentration in the antigenic carbohydrate complexes released from the cell walls of GBS types Ia, Ib and Ic. The following methods were used:

(1) Sialic acid release from whole GBS cells by the neuraminidase enzyme method. Streptococcal cells were cultured in 1 litre of Todd-Hewitt broth overnight at 37°C. The cells

were harvested and washed in two volumes (20 ml) of saline (0.85% w/v NaCl) by centrifugation at 1000g for 15 min at 4°C. The bacteria were freeze-dried overnight, weighed, and resuspended in water to a final concentration of 100 mg of cells per 1 ml of water.

Sialic acid was extracted from samples of whole cells by neuraminidase enzyme (P9D4). This enzyme was prepared according to the method described by Fraser (1975) and was a dialysed, Seitz filtered culture supernate of Clostridium perfringens strain L2A(b) grown for 48h at 37°C in proteose peptone water (PPW5 broth consisting of 5% Proteose Peptone, Difco, code 0120, and 0.5% w/v NaCl in water, adjusted to pH 7.0). Before use the enzyme was dissolved in 0.1M sodium acetate buffer, pH 5.1 in the ratio of 1 part enzyme to 1.5 parts buffer.

To perform the test, 0.25 ml enzyme in buffer was added to the sample of cells (0.25 ml of 100 mg/ml of cells in water) and release of sialic acid was effected by incubation for prescribed periods up to 24h at 37°C. Free sialic acid was determined by the method of Aminoff (1961) which is described later. Also included in the test was an enzyme control of 0.25 ml enzyme in buffer; a substrate control of 0.25 ml of cells in suspension with 0.25 ml of acetate buffer without enzyme, and a sialic acid standard (0.25 ml of 40 µg/ml synthetic sialic acid, Koch Light Laboratory Ltd, Colnbrook, U.K.).

(2) Sialic acid release from whole GBS cells by acid hydrolysis. GBS cells at a concentration of 100 mg/ml were prepared as in method (1). On this occasion the release of sialic

acid from cells was performed by hydrolysis using 0.02M HCl, and 0.25 ml of acid was added to 0.25 ml of cellular suspension. Samples were incubated for periods up to 24h in a water-bath at 80°C. Controls for each test were 0.25 ml of sialic acid (40 µg/ml) with 0.25 ml of 0.02M HCl incubated for 0h and 24h at 80°C, and 0.25 ml of cells with 0.25 ml water incubated for 0h and 24h at 80°C.

(3) Sialic acid release from SDS-purified GBS cell walls. Cell walls from each of the GBS serotypes were analysed to determine sialic acid content. Hydrolysis by 0.02M HCl (0.25 ml) of the cell wall samples (0.25 ml of 40 mg/ml cell walls in water) was performed by incubation up to 24h at 80°C to release sialic acid. Controls were included as before.

(4) Assay of TCA-extracted carbohydrates for sialic acid. Carbohydrate samples extracted from the cell walls were hydrolysed with 0.02M HCl using the method described above to release bound sialic acid. Unheated carbohydrate samples were also assayed to detect any free sialic acid present.

Once the carbohydrate samples had been separated and purified by passing through the DEAE-cellulose column, each sample collected was assayed for both free and bound sialic acid.

(5) Sialic acid release from residual cell walls after carbohydrate extraction. To ascertain that all sialic acid had been removed from the peptidoglycan component of the cell walls by the procedures used to extract carbohydrate, samples (0.25 ml) of residual cell walls (40 mg/ml) in water were hydrolysed in 0.02M HCl as previously described.

Detection of sialic acid

The method employed to detect free sialic acid was based on the thiobarbituric acid technique of Aminoff (1961). On completion of the procedures to extract sialic acid, 0.25 ml of 0.025M periodic acid in 0.125M sulphuric acid pH 1.2 (Aminoff's Reagent 1) was added to each of the test tubes containing samples and controls, and incubated in a 37°C water-bath for 30 min. The tubes were removed from the water-bath and 0.2 ml of 2% (w/v) sodium arsenite in 0.5M HCl (Reagent 2) was added. Tubes were shaken for approximately 1 min, or until the sample became colourless. Reagent 3 (2 ml of 0.1M 2-thiobarbituric acid in 0.1M NaOH, pH 9.0) was added and tubes were transferred to a boiling water bath for 7.5 min. The tubes were removed from the bath and cooled under a running tap. Finally, 4 ml of butan-1-ol containing 5% (v/v) 12M HCl was added to all tubes and the contents were vortexed until they had a 'milky' appearance. To achieve complete separation of the butanol and water phases, tubes were centrifuged at 800g for 1h at room temperature. The butanol layer was carefully transferred to 4 ml disposable plastic cuvettes and optical density readings at a wavelength of 549nm in a Pye-Unicam SP600 spectrophotometer were obtained. Some samples were subjected to scanning of their absorption spectra in a Pye-Unicam SP8000 spectrophotometer.

Results

(1) GBS serotype Ia

Cell wall and carbohydrate preparations

The yield of SDS-purified cell walls from 6 litres of type Ia cells was 210 mg. Treatment of cell walls (75 mg) with trichloroacetic acid extracted 2.14 mg of carbohydrate (i.e. 2.85 mg carbohydrate/100 mg of cell walls). Treatment of walls (50 mg) by hydrochloric acid produced 0.08 mg of carbohydrate (0.16 mg carbohydrate per 100 mg of cell walls); and hydrolysis of walls (60 mg) by sodium hydroxide produced 0.82 mg of carbohydrate (1.37 mg carbohydrate per 100 mg of cell walls).

These values were obtained by performing the phenol/sulphuric acid carbohydrate assay of Dubois et al. (1956) which does not detect amino-sugars or alditols. A standard sample of sialic acid was also not detected by this method.

Crossed immunoelectrophoresis (CIE) of cell wall antigens

Preliminary studies indicated that optimum antigen concentration for electrophoresis was 10 µl of carbohydrate at a concentration of 1 mg/ml in water, estimated by the phenol/sulphuric acid carbohydrate assay. Optimum antiserum concentration to obtain clear precipitin lines in the second dimension of CIE was 0.5 ml of antiserum in 3 ml of agarose.

Electrophoresis of the Ia antigens extracted by the three hydrolysis techniques was performed with antisera raised against the five main serotypes of GBS, and with commercial (Wellcome)

grouping antiserum. Fig. 5.6 shows the reaction between the TCA-prepared antigen with Ia type-specific antiserum. Two distinct precipitin lines can be observed.

Fig. 5.7 shows the reaction between the HCl-antigen and the same Ia antiserum. Two definite precipitin lines are seen, one of which has a double peak.

In Fig. 5.8 the NaOH-antigen was reacted with Ia antiserum and the precipitin lines (2) are similar to those in Fig. 5.6.

Figs. 5.9, 5.10 and 5.11 illustrate the results of electrophoresis of the three Ia antigens reacted with Ib type-specific antiserum. In each case single precipitin lines were produced which were similar to each other. The three gel slides were electrophoresed together under the same conditions and time, and because the distance between the apex of each precipitin peak and the origin of the antigen in the first dimension was identical, it can be said that these precipitin lines were the products of identical antigens.

Figs. 5.12, 5.13 and 5.14 show the precipitin reaction between the three Ia antigens and Ic type-specific antiserum. As in the previous group of slides, these precipitin lines are similar to each other.

No reaction was observed between the Ia antigens and type-specific antisera for GBS types II and III.

Figs. 5.15, 5.16 and 5.17 are similar and each shows two definite precipitin lines formed by the reaction between the Ia antigens and the commercial grouping antiserum. Limited information from Wellcome Reagents Ltd indicated that this antiserum had been prepared using a GBA Ia strain.

Purification and separation of TCA-carbohydrate antigens

The TCA-extracted cell wall carbohydrate antigens of GBS type Ia were subjected to DEAE-cellulose chromatography and eluted with increasing molarities of pyridinium acetate as previously described. Analysis from the column detected four carbohydrate fractions (see Fig. 5.18). Fraction 1 was eluted between 0.28M and 0.35M pyridinium acetate buffer, fraction 2 was eluted between 0.36M and 0.42M buffer, fraction 3 was eluted between 0.61M and 0.68M buffer, and fraction 4 was eluted between 0.96M and 1.0M pyridinium acetate buffer.

Crossed immunoelectrophoresis of purified carbohydrate fractions

Each of the four carbohydrate fractions collected from the DEAE-cellulose column were adjusted to a concentration of 1 mg/ml by the phenol/sulphuric carbohydrate assay and tested for antigenic activity by running with type-specific antisera in CIE.

Fraction 1 and 4 did not react with type-specific Ia antiserum. Fig. 5.19 shows the precipitin reaction between fraction 2 and Ia antiserum, and Fig. 5.20 shows the reaction between fraction 3 and Ia antiserum. When fractions 2 and 3 were run together in CIE with Ia antiserum, a gel slide with two precipitin lines was produced, similar to the reaction between the whole, unpurified Ia antigen and Ia antiserum (Fig. 5.6). Examination of the reactions between the two fractions and heterologous type-specific antisera revealed that fraction 2 reacted with Ib antiserum to produce a single precipitin line (Fig. 5.21), similar to the unpurified Ia carbohydrate - Ib antiserum reaction (Fig. 5.9);

but did not react with Ic antiserum. Fraction 3 did not react with Ib antiserum, but a single precipitin line was observed with Ic antiserum (Fig. 5.22). This was similar to the precipitin reaction between unpurified Ia antigen and Ic antiserum (Fig. 5.12).

To summarise these results, carbohydrate extracted from Ia cell walls contained two antigenic polymers which reacted with Ia type-specific antiserum and was visualised by CIE. The two polymers were separated by ion-exchange chromatography. One of the polymers reacted with Ia and Ib antiserum while the other reacted with Ia and Ic antiserum.

Chemical analysis of Ia carbohydrate fractions

Chemical analysis of fractions 1, 2 and 3 hydrolysed in 2M HCl for 4h at 100°C was performed in the first instance by descending paper chromatography (Fig. 5.23). Fraction 1 contained rhamnose and probably glucosamine, fraction 2 contained rhamnose, galactose, glucose and glucosamine, and fraction 3 consisted of only one sugar which was not clearly identified but was either ribose or rhamnose.

These sugars were converted to alditol acetates and identified and quantitated by gas-liquid chromatography. Fig. 5.24 shows standards of galactose, glucose, glucosamine and galactosamine run on column OV 225. Rhamnose was not included in this chromatogram since this sugar was easily detected using the SP 2330 column. Fraction 1 was shown to contain rhamnose and glucosamine in the molar proportions 7:1 respectively (Fig. 5.25). Fraction 2 contained rhamnose, galactose, glucose and glucosamine in the

molar proportions 1:0.9:1:0.7 (Fig. 5.26). Fraction 3 appeared to contain only a small amount of rhamnose (Fig. 5.27).

Sialic acid determinations

Preliminary experiments to confirm the presence of sialic acid in Ia cells were carried out by heating samples (25 mg dry weight) of cells in 0.02M HCl at 80°C for periods up to 24h. Table 5.1 illustrates the quantity of sialic acid released from cells. Control samples of sialic acid subjected to the same conditions as the test samples showed that, after 4h heating in HCl, there was a 30% loss of sialic acid, and after 24h a 70% loss. The results in Table 5.1 and all subsequent analyses were corrected accordingly for destruction of sialic acid.

The concentration of free sialic acid in the unpurified carbohydrate extracted from Ia cell walls was 252 µg of sialic acid in 1 mg of carbohydrate.

Each sample eluted from the DEAE-cellulose column which separated the Ia carbohydrate into four fractions, was assayed for both free and bound sialic acid. Carbohydrate fraction 2 alone contained sialic acid and this is shown graphically in Fig. 5.28.

Phosphate analyses

Analysis of the four carbohydrate fractions for phosphate detected only trace amounts in all samples.

Crossed immunoelectrophoresis of Ia "Lancefield antigens"

The classical method to extract group and type-specific

antigens from whole GBS cells, serotype Ia, was performed as described previously. These antigenic complexes were electrophoresed with GBS group and type-specific antisera. Fig. 5.29 is the reaction between Ia Lancefield prepared group antigen with commercial grouping antiserum. One precipitin line (i) was observed, which is similar to the precipitin line produced between the cell wall fraction 2 sample with Ia and Ib antiserum (Fig. 5.19 and Fig. 5.21). The reaction between the Ia Lancefield-prepared type-specific antigen and Ia type-specific antiserum (Fig. 5.30) produced two main precipitin lines (ii and i) and two faint precipitin lines (iii and iv).

In Fig. 5.31 the Ia Lancefield type-specific antigen was reacted with Ib antiserum to produce two main precipitin lines (ii and i) and two faint lines (iv and v). Finally, Fig. 5.32 shows the reaction between Ia Lancefield type antigen and Ic antiserum. The two precipitin lines ii and i are clearly observed but are not accompanied by the fainter precipitin lines. No reaction occurred between the Ia antigens and antisera of types II and III.

(2) GBS serotype Ib

Cell wall and carbohydrate preparations

The yield of SDS-purified cell walls from 6-litres of GBS type Ib cells was 600 mg. Previous studies with serotype Ia cell walls had shown that significantly greater amounts of carbohydrate had been extracted from walls by hydrolysis in trichloroacetic acid (TCA). Accordingly, the TCA technique

only was used to extract Ib carbohydrate. The total carbohydrate extracted was approximately 6 mg (i.e. 1 mg of carbohydrate per 100 mg of cell walls).

Crossed immunoelectrophoresis (CIE) of cell wall antigens

Similar antigen and antiserum concentrations were used as in experiments with serotype Ia. Electrophoresis of the Ib cell wall antigen was performed with the homologous and heterologous GBS antisera.

Single precipitin lines were observed with Ia (Fig. 5.33), Ib (Fig. 5.34), Ic (Fig. 5.35) and the commercial grouping antiserum (Fig. 5.36). No reaction occurred between the Ib antigen and antisera of types II and III.

Purification of the TCA carbohydrate antigen

The Ib carbohydrate was purified by DEAE-cellulose column chromatography and eluted with increasing molarities of pyridinium acetate buffer as before.

Analysis of the samples from the column detected one carbohydrate fraction only, eluting between 0.05M and 0.2M pyridinium acetate (Fig. 5.37). Crossed immunoelectrophoresis of this fraction showed it to be immunologically similar to the unpurified extract.

Chemical analysis of the Ib carbohydrate fraction

Paper chromatography of the purified sample hydrolysed in 2M HCl at 100°C for 4h (S_1) resulted in the detection of glucosamine

and rhamnose, whereas hydrolysis in 4M HCl at 100°C for 18h (S_2) detected glucosamine only (Fig. 5.38).

Confirmation of these results was obtained by analysis of the derivitised hydrolysates by gas-liquid chromatography. Figure 5.39 shows standards of pentitol sugars run on column SP 2330. In Figure 5.40 the chromatogram on column SP 2330 of the 2M HCl hydrolysed derivitive (S_1) showed rhamnose, glucose and galactose, and one unknown peak. The retention time of this unknown sugar was similar to that of ribose, but co-injection of a ribose standard and the sample resulted in the formation of two peaks denoting non-identity. In Figure 5.41 the sample S_1 was chromatographed on column OV 225 and the two main peaks were rhamnose and glucosamine. Expressed as molar proportions the Ib antigen was shown to contain the following compounds: galactose (0.6), glucosamine (1.0), glucose (0.2), and rhamnose (1.9). The sialic acid concentration in the antigen is discussed below.

Sialic acid determinations

The release of sialic acid from 25 mg (dry weight) of Ib whole cells by the neuraminidase enzyme method and hot HCl method is illustrated in Table 5.2. The acid method was shown to release significantly greater amounts of sialic acid throughout the 24h extraction period in comparison to the enzyme method. For this reason, extraction by HCl was used for all subsequent analyses of sialic acid.

Analysis of 10 mg (dry weight) samples of SDS-purified Ib cell walls indicated that after 4h of acid treatment 120.0 µg of sialic acid had been released. Gradual destruction of sialic

then occurred so that at 24h only 81.0 µg was detected.

Following TCA treatment of the walls, the sialic acid content of the extract was 63 µg/mg of carbohydrate; of this 30.5 µg was in the bound form. The relative overall proportion of sialic acid in the complete Ib antigen is shown in Table 5.3.

A final analysis for sialic acid was performed on the residual cell walls after TCA extraction, to determine if complete removal had been obtained. It was found that from a sample (10 mg) of walls heated in HCl for 4h, 3.9 µg of sialic acid was released, signifying almost total extraction during the TCA hydrolysis.

The standard trace produced by sialic acid obtained from the Pye-Unicam SP8000 spectrophotometer is shown in Fig. 5.42.

Phosphate analysis

Trace amounts of phosphate were detected in the Ib carbohydrate antigen.

Crossed immunoelectrophoresis of Ib 'Lancefield antigens'

The Ib group and type-specific antigens were prepared as described previously, and electrophoresed with the GBS antisera.

Fig. 5.43 shows two precipitin lines produced by reacting the Ib type-specific antigen with Ia antiserum. One of the lines (i) is the same as the precipitin line obtained after electrophoresis of the Ib cell wall antigen and Ia antigen. The second precipitin line was unique to the Ib antigen complex and was labelled (vi). Fig. 5.44 illustrates the Ib-Ib reaction, the two main precipitin lines were i and vi, and the adjacent fainter lines were iii and iv seen previously in the Ia antigen experiments.

CIE of the Ib type antigen with Ic antiserum (Fig. 5.45) resulted in one major precipitin line (i) with two fainter staining lines (iii and iv) on either side. No reaction occurred with antiserum of types II and III.

Electrophoresis of the Ib group-specific antigen with Ib typing serum resulted in three distinct precipitin lines (Fig. 5.46). In relation to the other reactions described, these lines are produced by antigens i, ii and iii. Reaction of the Ib group antigen with commercial GBS grouping serum resulted in one definite precipitin line (i) (Fig. 5.47).

(3) GBS serotype Ic

Cell wall and carbohydrate preparations

The yield of SDS-purified cell walls from 6 litres of GBS type Ic cells was 450 mg. The total carbohydrate extracted by TCA was approximately 4 mg (i.e. 0.9 mg of carbohydrate per 100 mg of cell walls).

Crossed immunoelectrophoresis (CIE) of cell wall antigens

CIE was performed with the Ic cell wall antigens and GBS antisera. In Fig. 5.48, the reaction of the Ic antigens with Ia antiserum produced two precipitin lines, whereas the Ic antigen with Ib and Ic antisera resulted in gel slides with just one precipitin reaction (Fig. 5.49 and Fig. 5.50). Comparison of these precipitin reactions with those of Ia cell wall antigens against the same antisera showed that the antigens extracted from Ic cell walls were immunologically identical to those from Ia cell walls. No reaction occurred with antisera of types II and III GBS.

Purification of the TCA-carbohydrate antigens

Purification and separation of the Ic cell wall antigens was performed by DEAE-cellulose column chromatography as before (Fig. 5.51). Two carbohydrate fractions were eluted, the first between 0.2M and 0.36M pyridinium acetate buffer, and the second fraction between 0.37M and 0.44M.

CIE of the two fractions (1 mg/ml carbohydrate) showed that they were both immunologically active; both fractions reacted with Ia antiserum (Fig. 5.52 and Fig. 5.53). Fraction 1 reacted with Ib antiserum to produce a precipitin line similar to that in Figure 5.52, and fraction 2 reacted with Ic antiserum similar to that in Figure 5.53.

Chemical analysis of the Ic carbohydrate fractions

Descending paper chromatography of fraction 1 hydrolysed in 2M HCl at 100°C for 4h indicated the presence of glucosamine, glucose, and rhamnose. Hydrolysed samples of fraction 2 contained glucosamine and glucose.

Analysis of the fraction 1 by GLC on columns of SP2330 and OV225 detected rhamnose, galactose and glucosamine (Fig. 5.54 and Fig. 5.55) in the molar proportions 12.4:1:1.7 respectively.

A trace amount of unknown sugar was also detected using column SP2330. This sugar had the same retention time as that found in the S_1 sample of the Ib carbohydrate.

Fraction 2 contained glucose, galactose (Fig. 5.56) and glucosamine (Fig. 5.57) in the molar proportions 1:2:1 respectively.

Sialic acid determinations

Analysis of the unpurified cell wall extract from type Ic cells detected 408 µg of sialic acid per milligram of carbohydrate, of which 378 µg was in the bound form. The sialic acid concentration in the purified Ic carbohydrate fractions is shown graphically in Fig. 5.51.

Phosphate analysis

Trace amounts of phosphate were detected in the Ic carbohydrate extract.

Crossed immunoelectrophoresis of 'Lancefield' Ic antigens

Immunoelectrophoresis of the 'Lancefield' prepared grouping antigen from Ic cells with commercial grouping antiserum resulted in the formation of one precipitin line which was identified as line i shown in the previous experiments.

The reaction between Ic type-specific antigen and Ia antiserum (Fig. 5.58) produced two precipitin lines (vii and viii). Precipitin line vii was also seen in the Ic antigen-Ib antiserum reaction, and both lines vii and viii, in addition to line i, were observed in the homologous Ic reaction (Fig. 5.59).

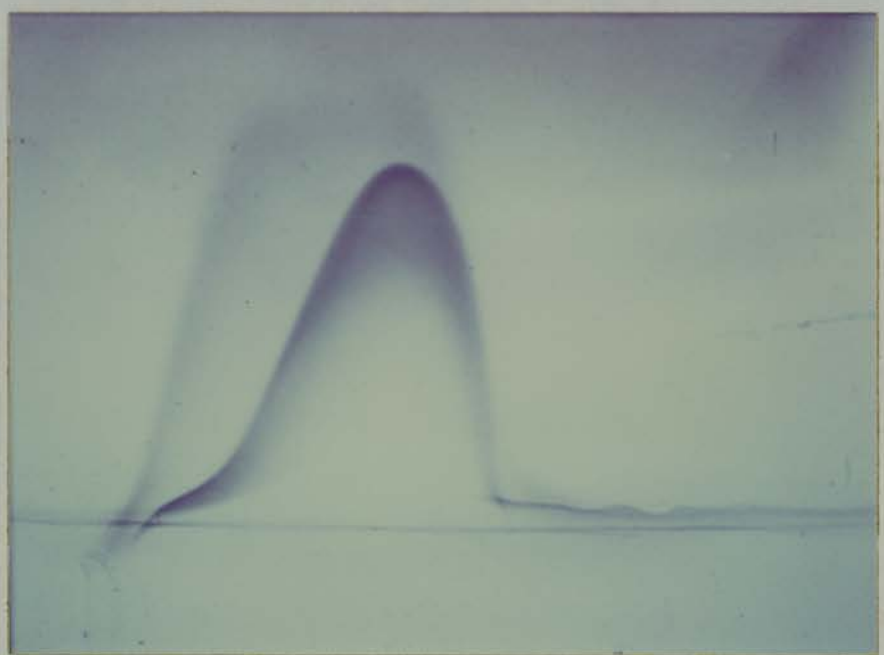


Fig. 5.6 CIE of Ia (TCA-prepared) cell wall antigens with Ia type-specific antiserum.

Fig. 5.7 CIE of Ia (HCl-prepared) cell wall antigens with Ia type-specific antiserum.

Fig. 5.8 CIE of Ia (NaOH-prepared) cell wall antigens with Ia type-specific antiserum.

Fig. 5.9 CIE of Ia (TCA-prepared) cell wall antigens with Ib type-specific antiserum.

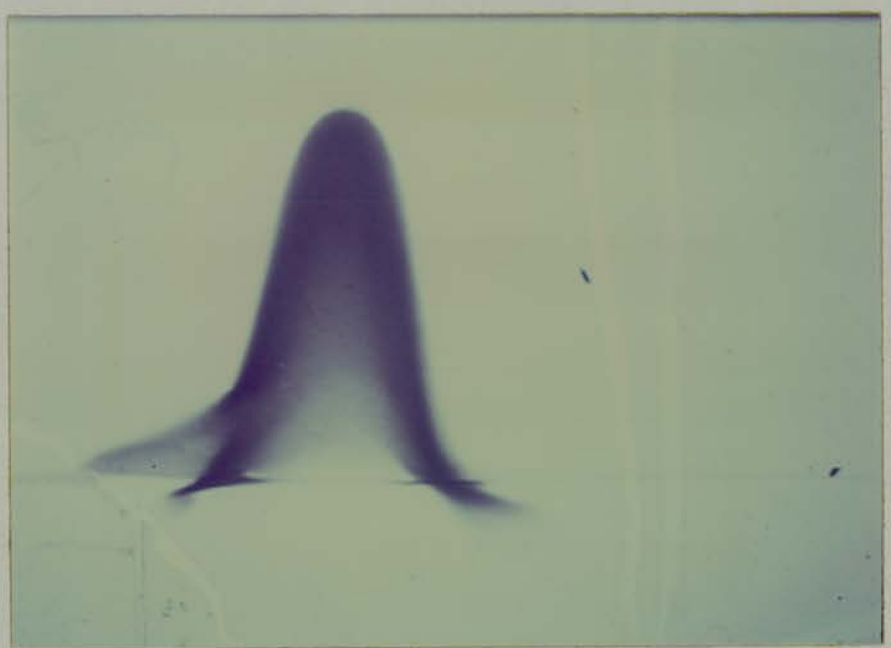


Fig. 5.10 CIE of Ia (HCl-prepared) cell wall antigens with Ib type-specific antiserum.

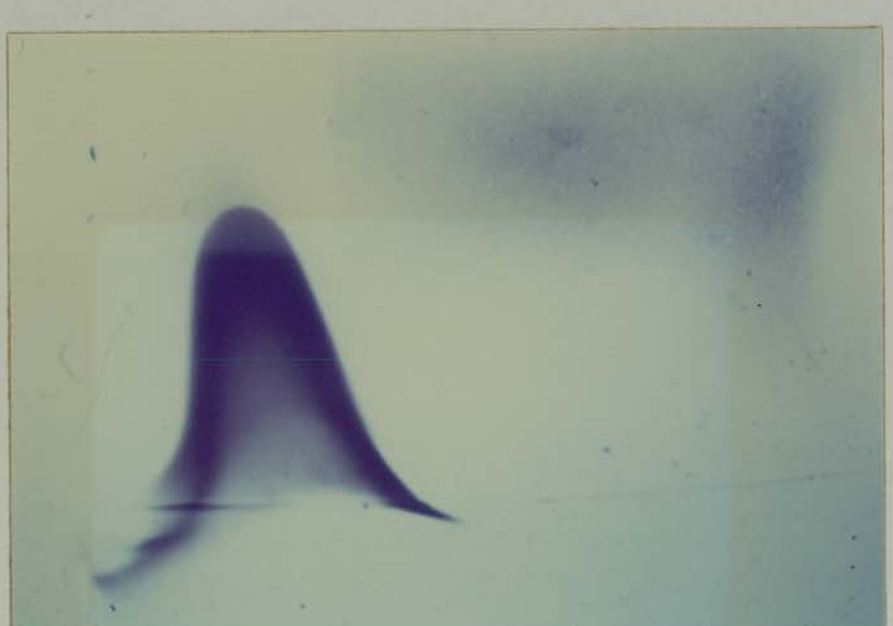


Fig. 5.11 CIE of Ia (NaOH-prepared) cell wall antigens with Ib type-specific antiserum.

Fig. 5.12 CIE of Ia (TCA-prepared) cell wall antigens with Ic type-specific antiserum.

Fig. 5.13 CIE of Ia (HCl-prepared) cell wall antigens with Ic type-specific antiserum.

Fig. 5.14 CIE of Ia (NaOH-prepared) cell wall antigens with Ic type-specific antiserum.

Fig. 5.15 CIE of Ia (TCA-prepared) cell wall antigens with commercial group B antiserum.

Fig. 5.16 CIE of Ia (HCl-prepared) cell wall antigens with commercial group B antiserum.

Fig. 5.17 CIE of Ia (NaOH-prepared) cell wall antigens with commercial group B antiserum.

Fig. 5.18 Separation of the TCA-prepared Ia cell wall extract from a DEAE-cellulose column by increasing molarities of pyridinium acetate buffer (0.1M).

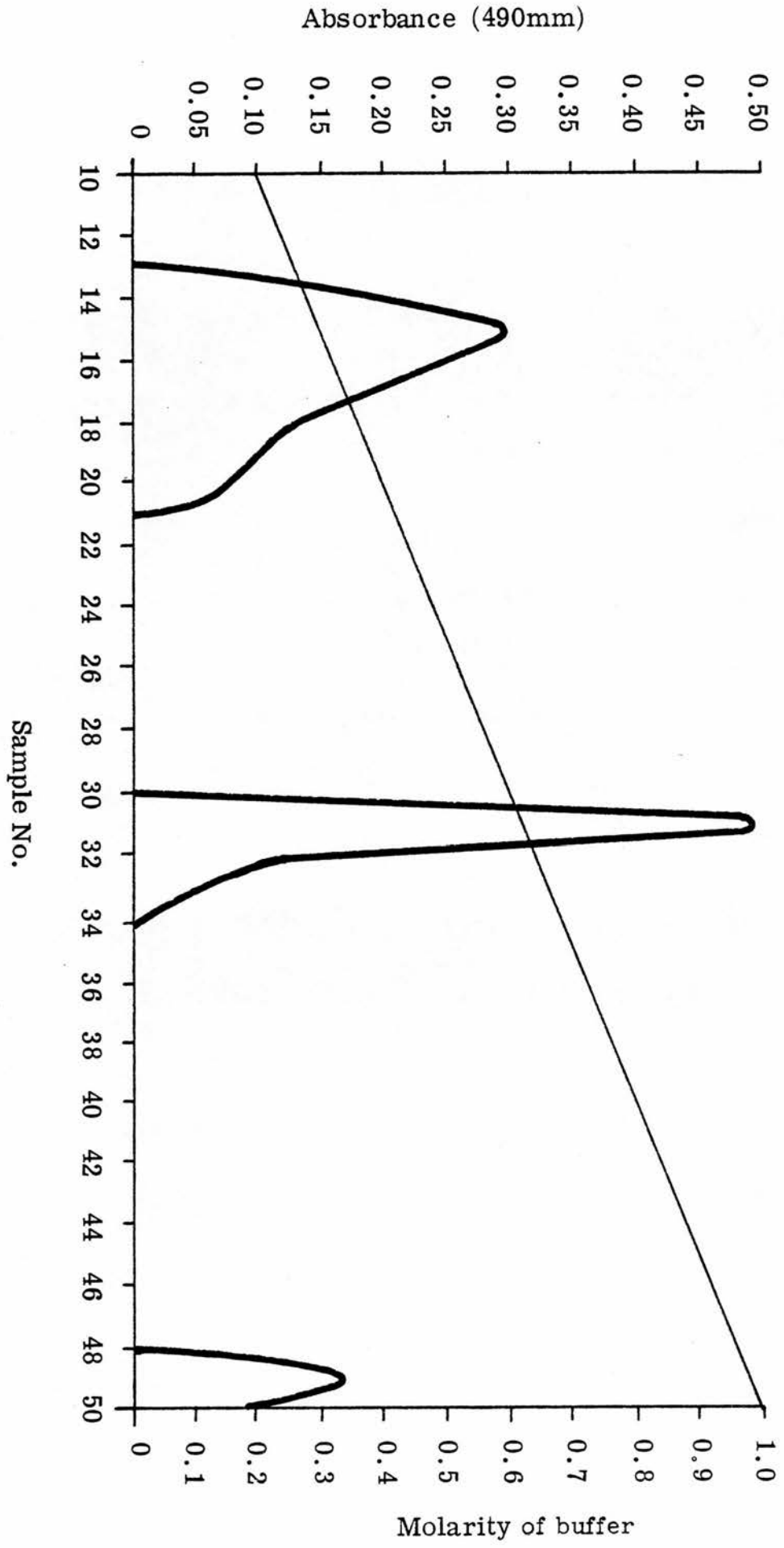

Absorbance (490mm)
0 0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50
10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46 48 50
Sample No.
0 0.1 0.2 0.3 0.4 0.5 0.6 0.7 0.8 0.9 1.0
Molarity of buffer

Fig. 5.19 CIE of Ia fraction 2 with Ia type-specific antiserum.

Fig. 5.20 CIE of Ia fraction 3 with Ia type-specific antiserum.

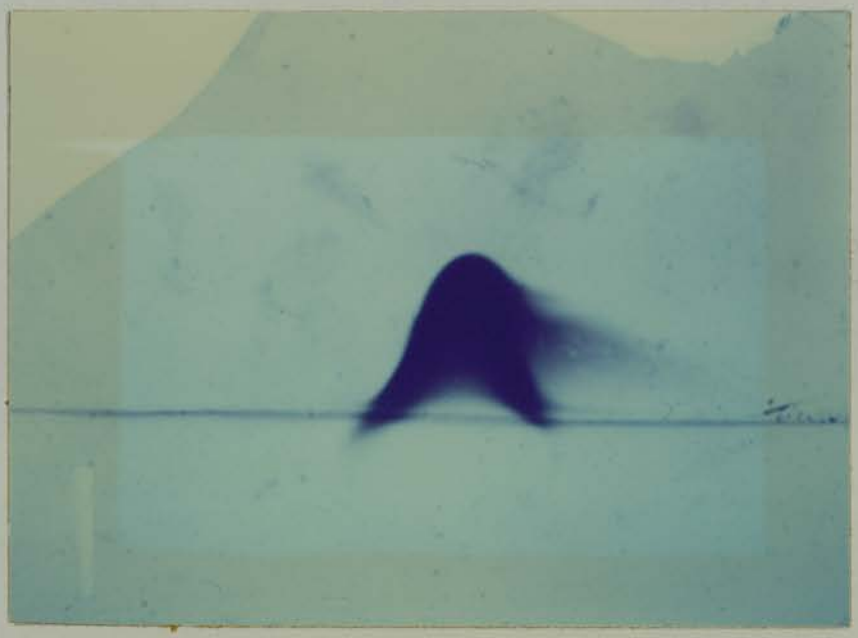


Fig. 5.21 CIE of Ia fraction 2 with Ib type-specific antiserum.

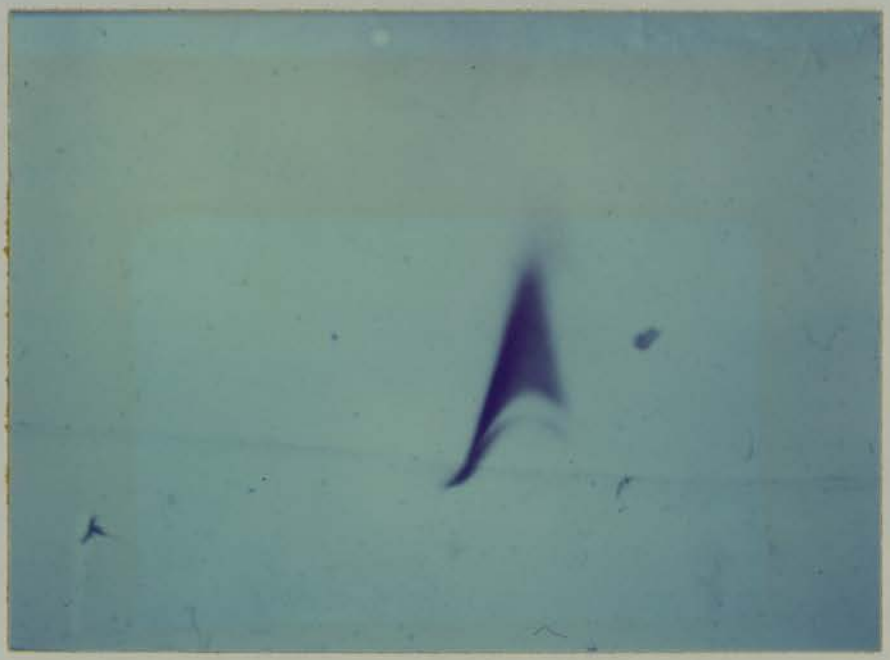


Fig. 5.22 CIE of Ia fraction 3 with Ic type-specific antiserum.

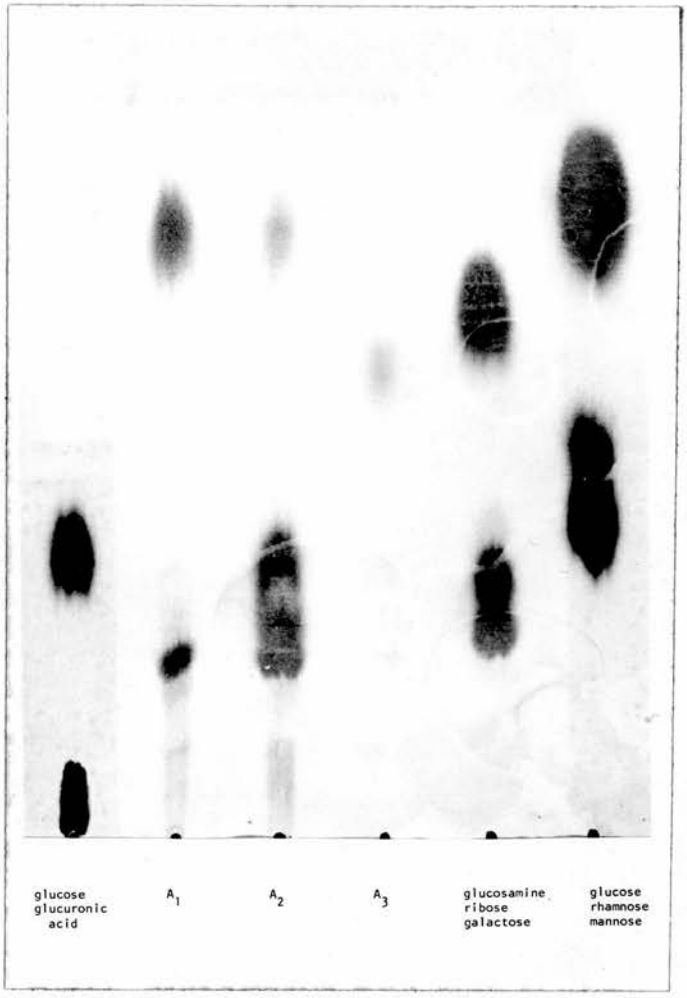


Fig. 5.23 Paper chromatogram of Ia acid-hydrolysed (2M HCl for 4h at 100°C) fractions 1, 2 and 3. Staining by the alkaline silver nitrate reagents of Trevelyan et al., 1950.

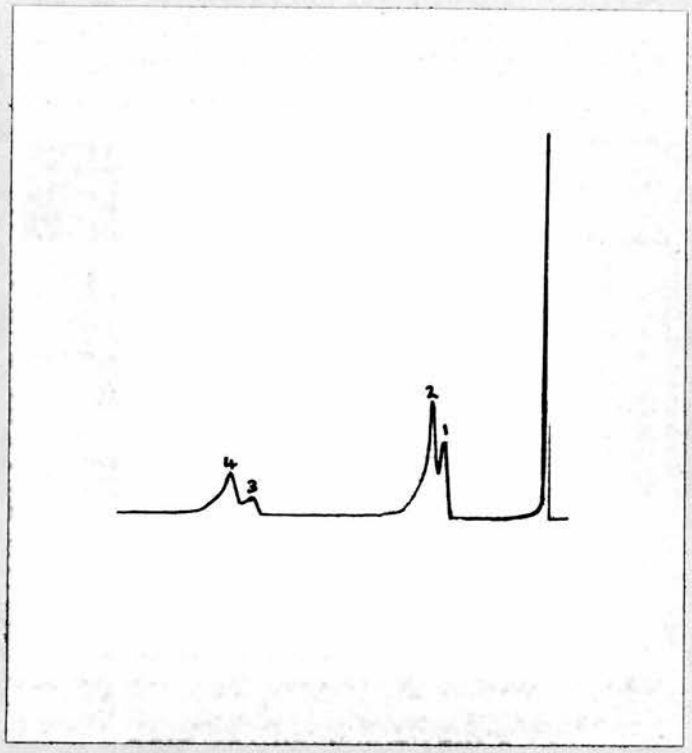


Fig. 5.24 GLC on column OV225 of alditol acetate derivatives of acid hydrolysates of galactose (1), glucose (2), glucosamine (3) and galactosamine (4) standards.

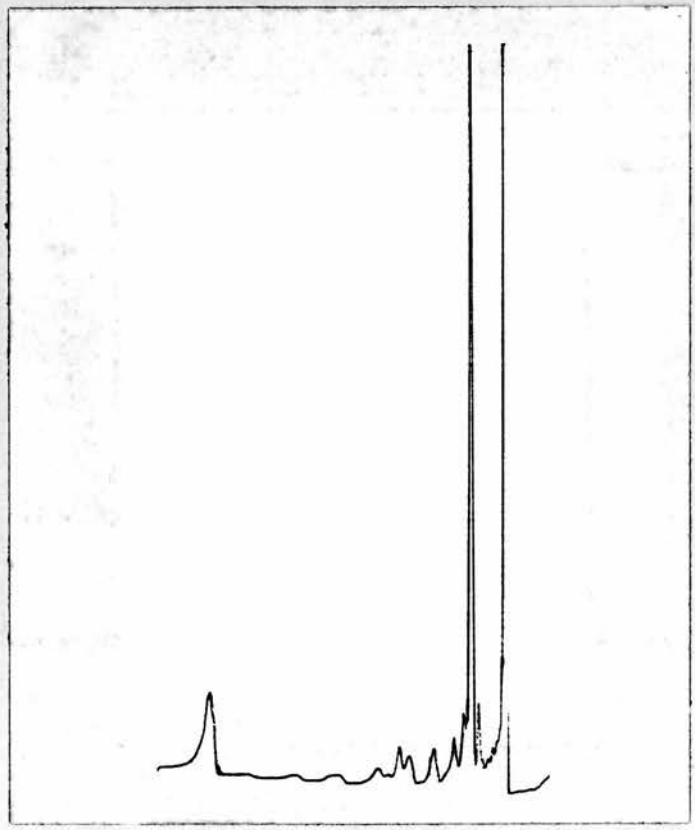


Fig. 5.25 GLC on column OV225 of alditol acetate derivatives of acid hydrolysed Ia fraction 1 indicating rhamnose and glucosamine.

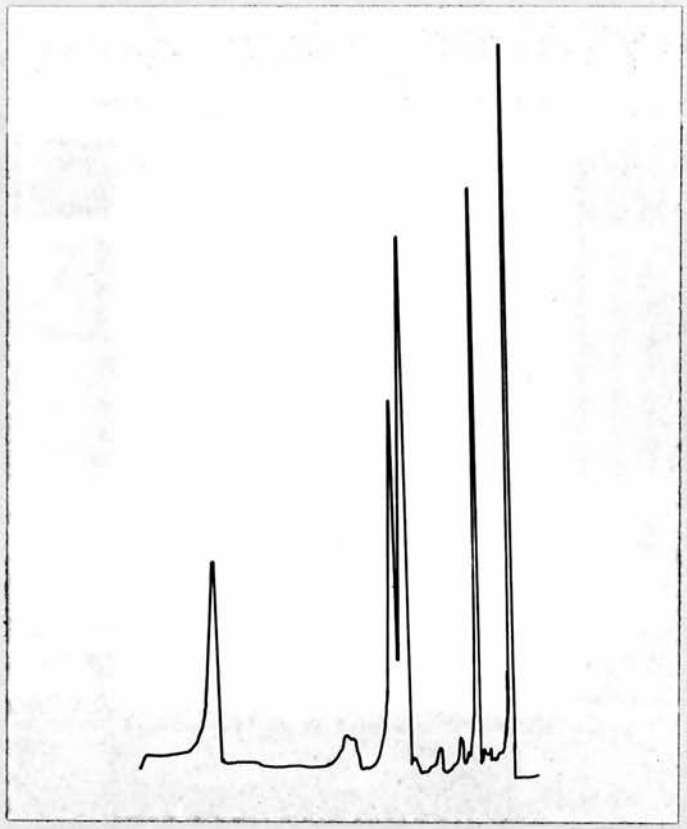


Fig. 5.26 GLC on column OV225 of alditol acetate derivatives of acid hydrolysed Ia fraction 2 indicating rhamnose, galactose, glucose and glucosamine.

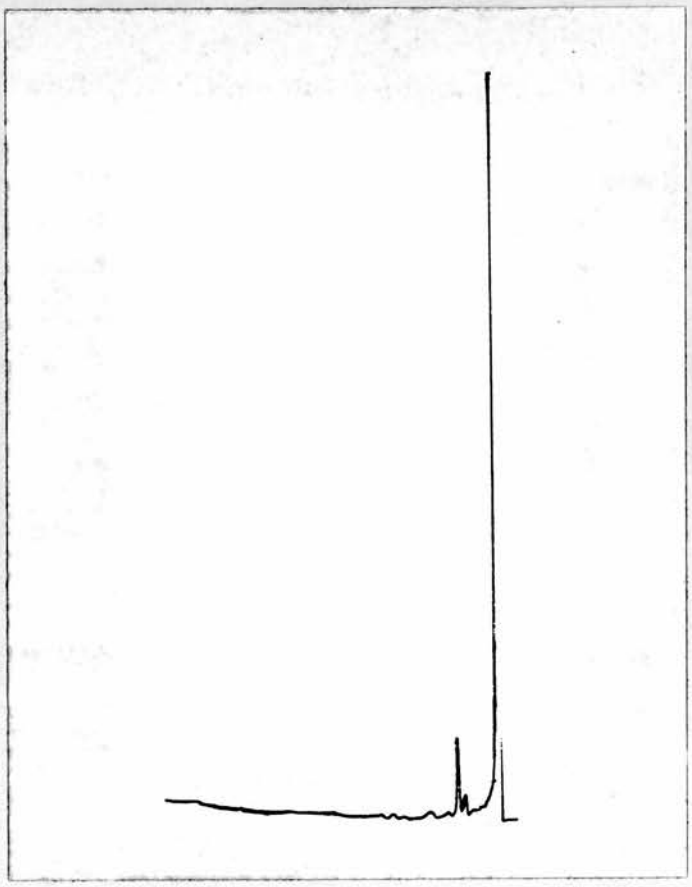


Fig. 5.27 GLC on column OV225 of alditol acetate derivatives of acid hydrolysed Ia fraction 3 indicating a trace amount of rhamnose.

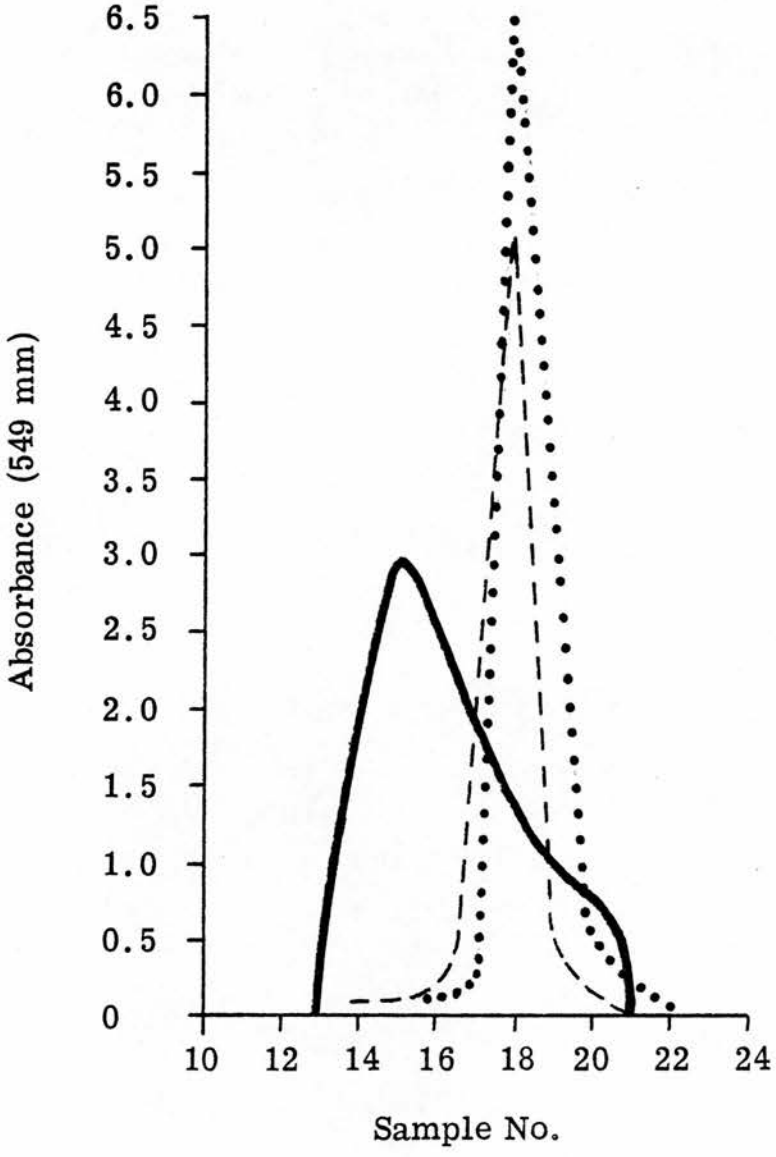


Fig. 5.28 Ia carbohydrate fractions 1 and 2 (▬▬). Samples containing free (• • • •) and bound (– – – –) sialic acid are indicated. Samples were eluted with increasing molarities (0-1M) of pyridinium acetate buffer from a DEAE-cellulose column.

Fig. 5.29 CIE of "Lancefield-prepared" group antigen of Ia cells with commercial group B antiserum.

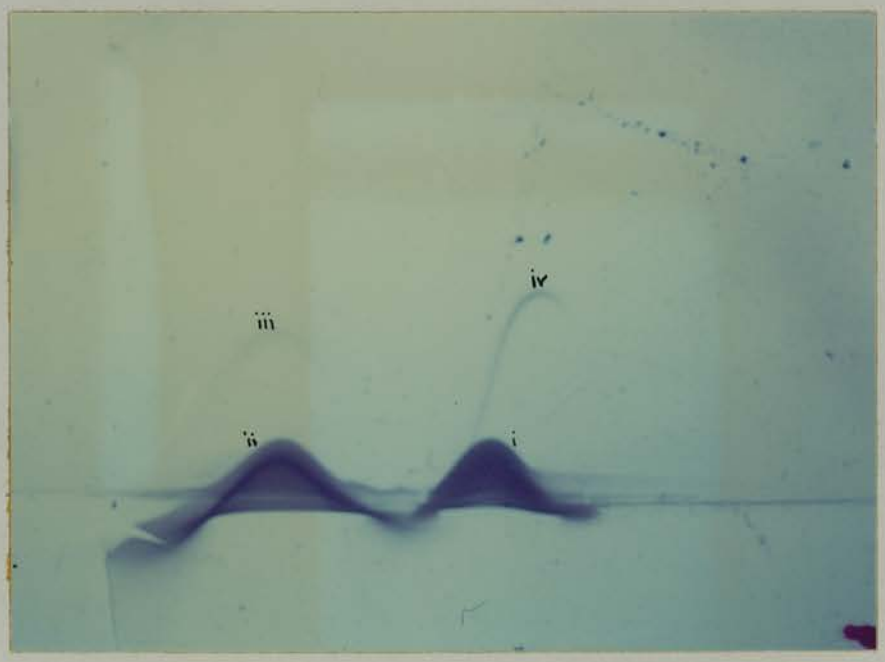


Fig. 5.30 CIE of "Lancefield-prepared" type Ia antigens with Ia type-specific antiserum. Four precipitin lines (i, ii, iii and iv) are labelled.

Fig. 5.31 CIE of "Lancefield-prepared" type Ia antigens with Ib type-specific antiserum. Four precipitin lines (i, ii, iv, v) are labelled.

Fig. 5.32 CIE of "Lancefield-prepared" type Ia antigens with Ic type-specific antiserum. Two precipitin lines (i, ii) are labelled.

Table 5.1 The release of sialic acid from 25 mg of Ia cells over a 24h period.

Time (hour)	Sialic acid (μg)
1	-
2	4.7
4	46.1
24	63.1

Fig. 5.33 CIE of Ib cell wall antigens with Ia type-specific antiserum.

Fig. 5.34 CIE of Ib cell wall antigens with Ib type-specific antiserum.

Fig. 5.35 CIE of Ib cell wall antigens with Ic type-specific antiserum.

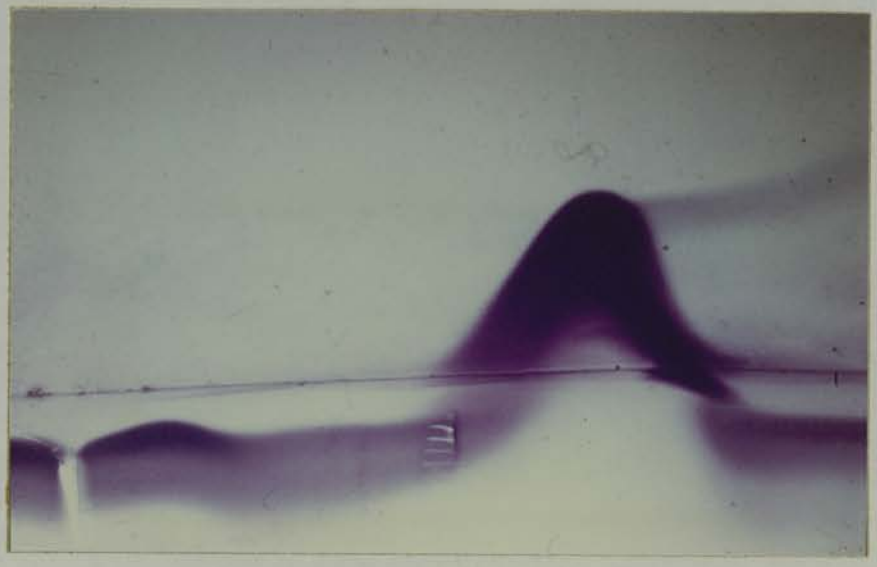
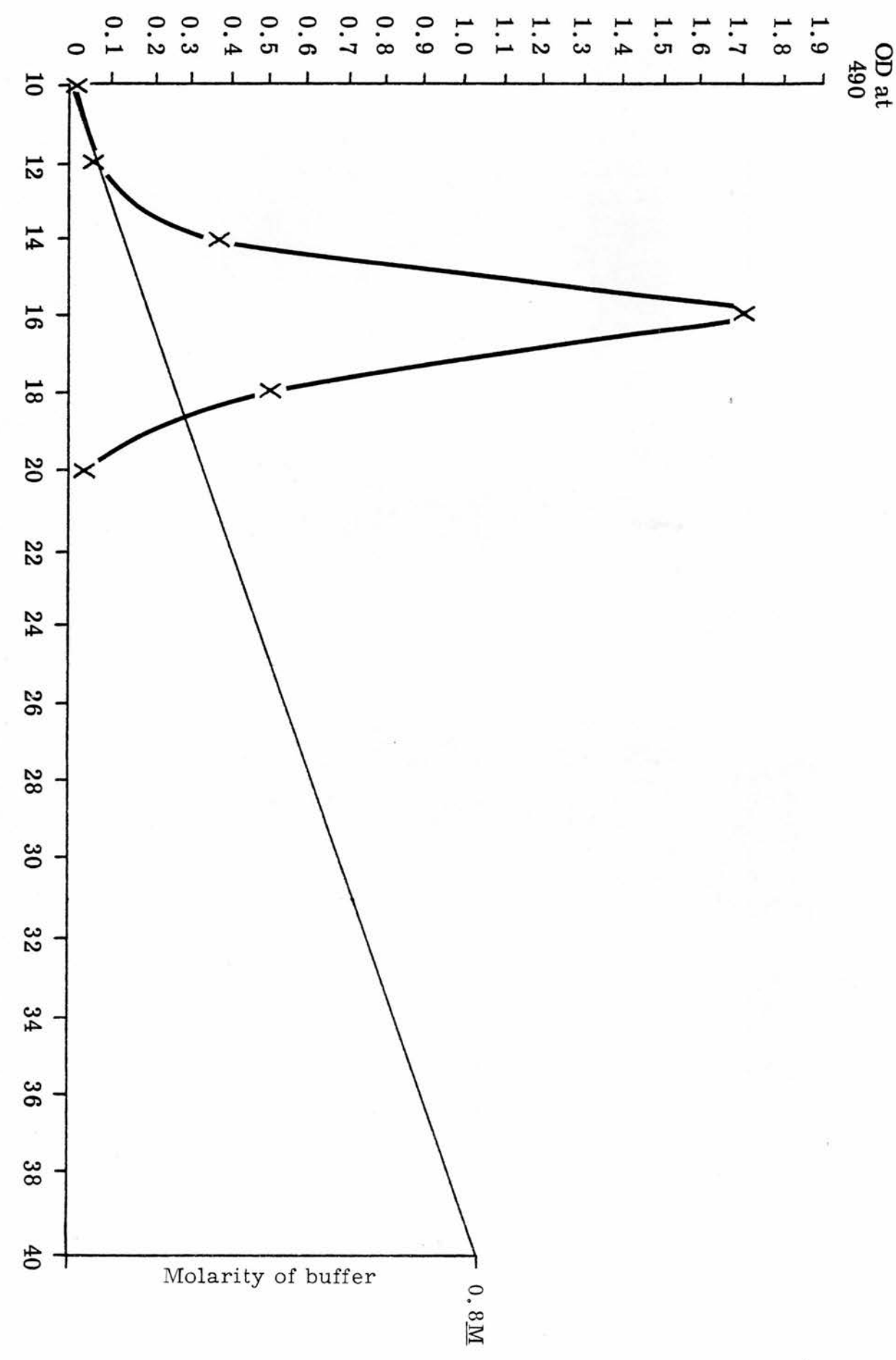


Fig. 5.36 CIE of Ib cell wall antigens with commercial group B antiserum.

Fig. 5.37 Purification of the lb cell wall TCA-extract by DEAE-cellulose column chromatography. Fraction was eluted by increasing molarities of pyridinium acetate buffer (0-1M).


OD at 490
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
Molarity of buffer
0.8M

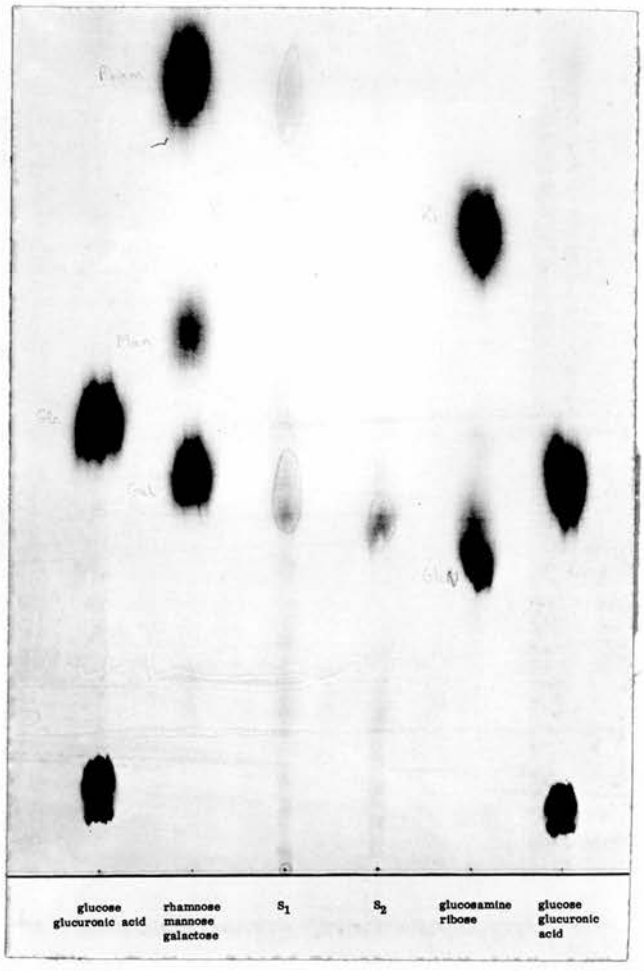


Fig. 5.38 Paper chromatogram of Ib fractions (S_1 = 2M HCl hydrolysis for 4h at 100°C; S_2 = 4M HCl hydrolysis for 18h at 100°C). Staining by the alkaline silver nitrate reagents of Trevelyan et al., 1950.

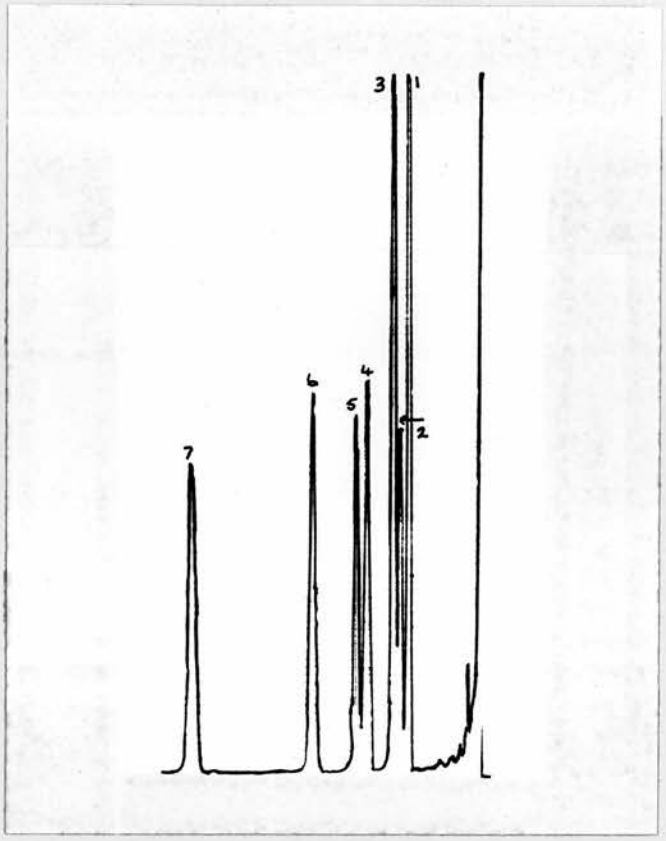


Fig. 5.39 GLC on column SP2330 of alditol acetate derivatives of acid hydrolysates of pentitol standards; deoxyribose (1), rhamnose (2), fucose (3), ribose (4), arabinose (5), xylose (6), glucose (7).

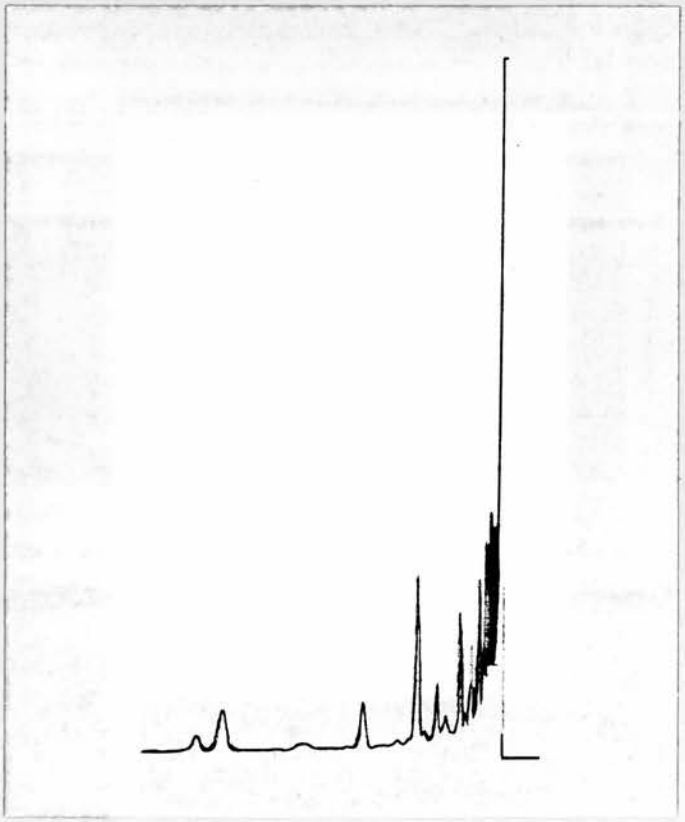


Fig. 5.40 GLC on column SP2330 of alditol acetate derivatives of the acid hydrolysed Ib derivative (S_1) indicating rhamnose, an unknown sugar, glucose and galactose.

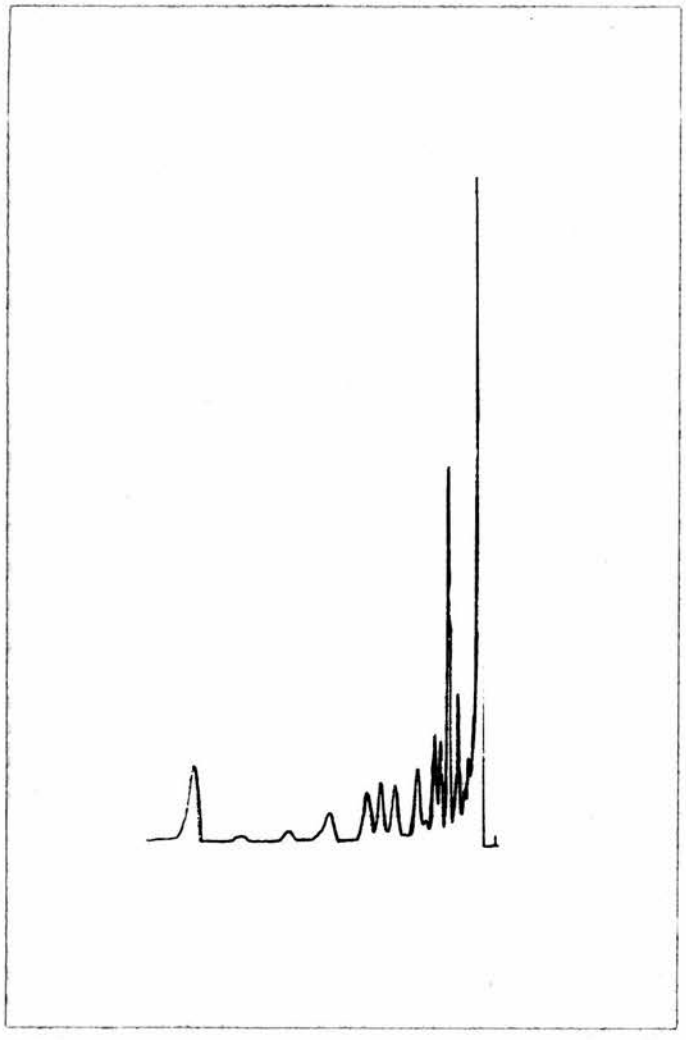


Fig. 5.41 GLC on column OV225 of alditol acetate derivatives of the acid hydrolysed Ib derivative (S_1) indicating rhamnose and glucosamine.

Fig. 5.42 Absorbance of a sialic acid standard (1) and hydrolysed Ib cell wall extract (2) measured in a Pye-Unicam SP8000 scanning spectrophotometer.

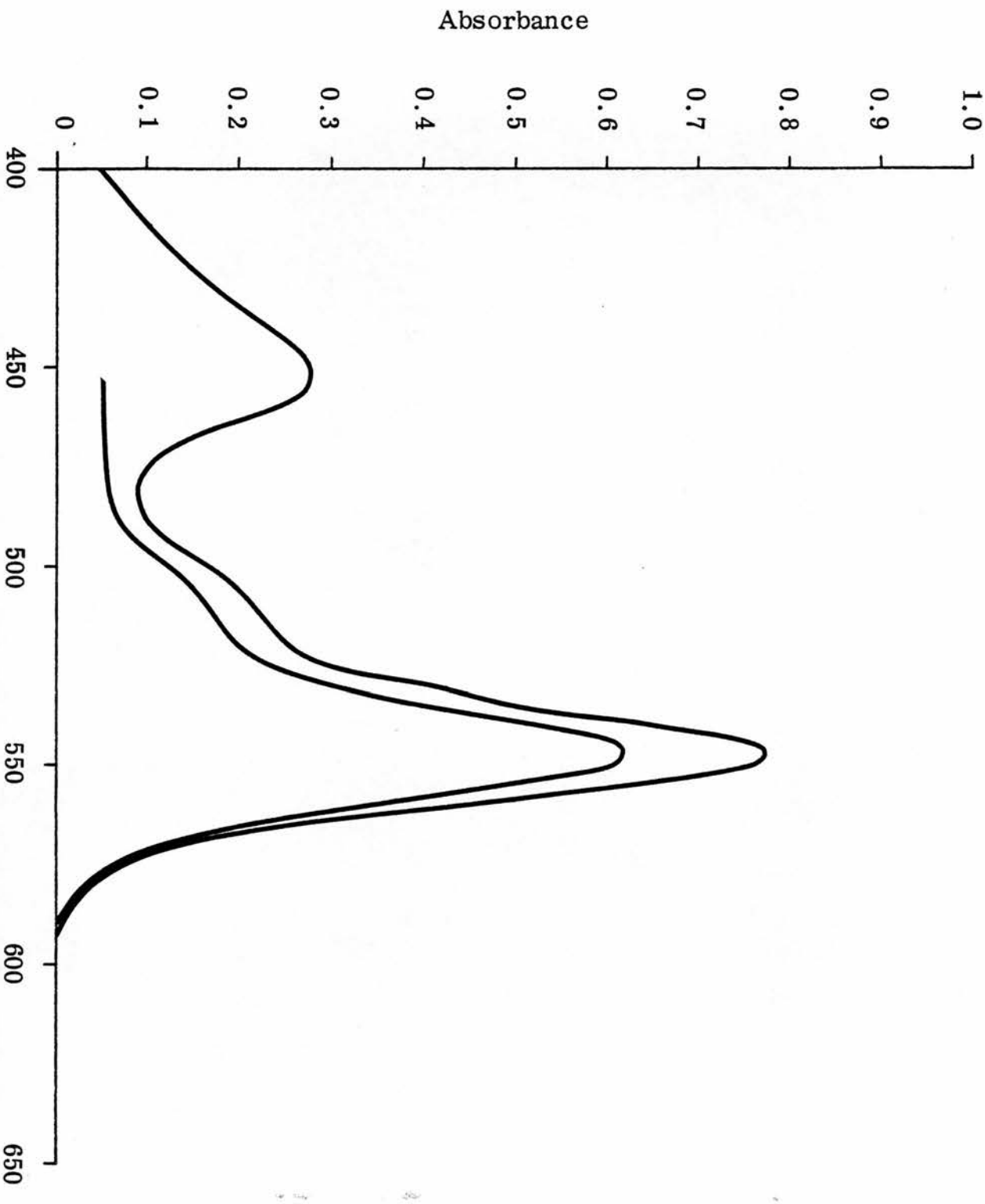

Absorbance
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1.0
400
450
500
550
600
650

Fig. 5.43 CIE of "Lancefield-prepared" type Ib antigens with Ia type-specific antiserum.

Fig. 5.44 CIE of "Lancefield-prepared" type Ib antigens with Ib type-specific antiserum.

Fig. 5.45 CIE of "Lancefield-prepared" type Ib antigens with Ic type-specific antiserum.

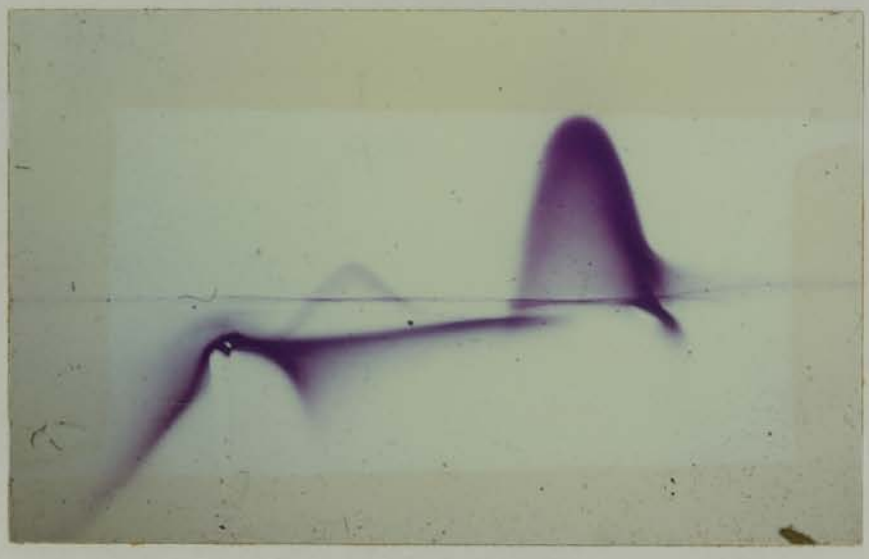


Fig. 5.46 CIE of "Lancefield-prepared" group antigens of Ib cells with Ib type-specific antiserum.

Fig. 5.47 CIE of "Lancefield-prepared" group antigens of Ib cells with commercial group B antiserum.

Table 5.2 Release of sialic acid from 25 mg samples of Ib whole cells.

Time (hours)	Sialic acid (µg)	
	Enzyme method	Acid method
1	3.0	5.4
2	5.7	9.2
4	9.0	18.1
24	7.0	76.7

Table 5.3 Chemical composition of cell wall extract of type Ib GBS, strain H36B.

Chemical components	Molar proportions
Galactose	0.6
Glucosamine	1.0
Glucose	0.2
Rhamnose	1.9
Sialic acid	0.03

Fig. 5.48 CIE of Ic cell wall antigens with Ia type-specific antiserum.

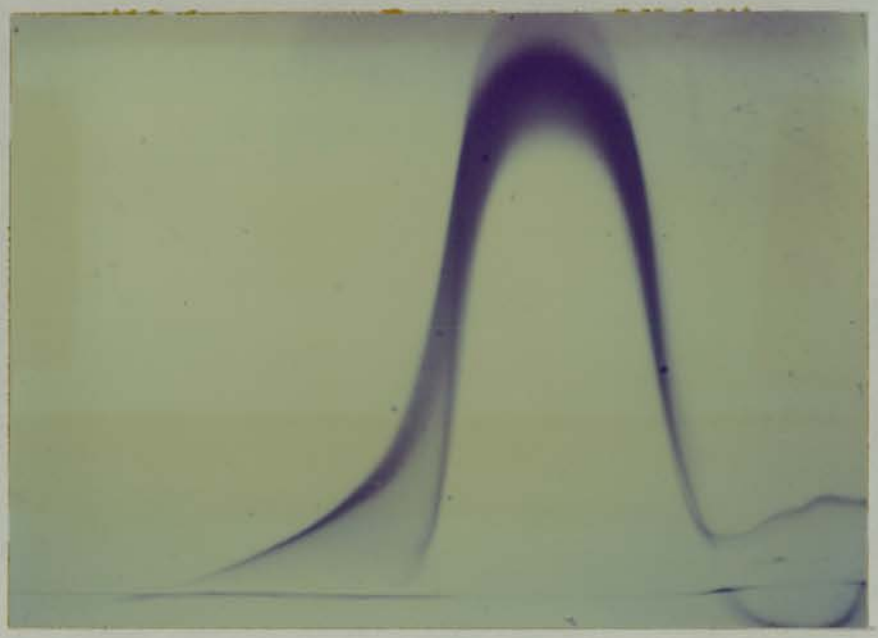


Fig. 5.49 CIE of Ic cell wall antigens with Ib type-specific antiserum.

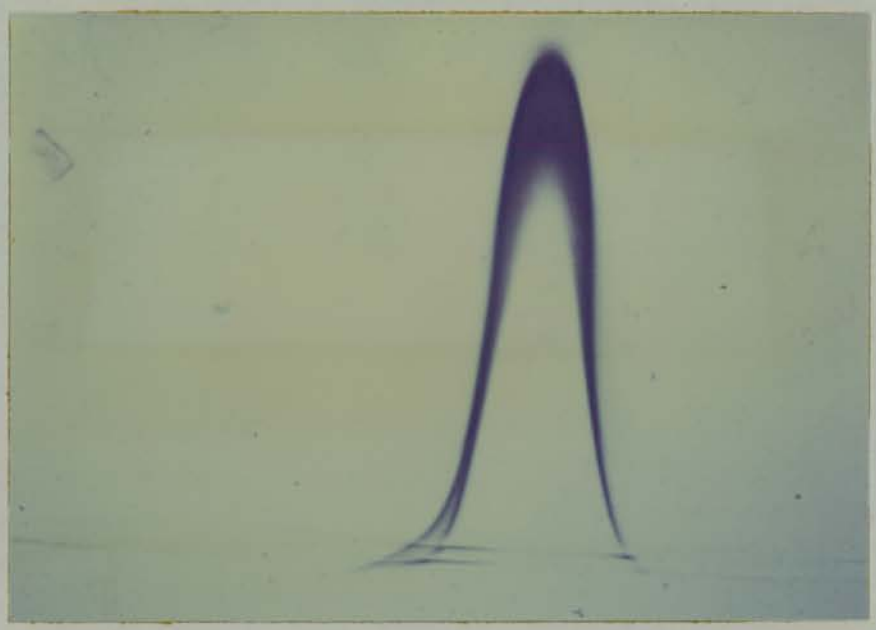


Fig. 5.50 CIE of Ic cell wall antigens with Ic type-specific antiserum.

Fig. 5.51 Separation of the Ic cell wall extract from a DEAE-cellulose column by increasing molarities of pyridinium acetate buffer (0-1M) resulted in two distinct fractions (1 and 2). Sialic acid concentration in each sample is denoted by

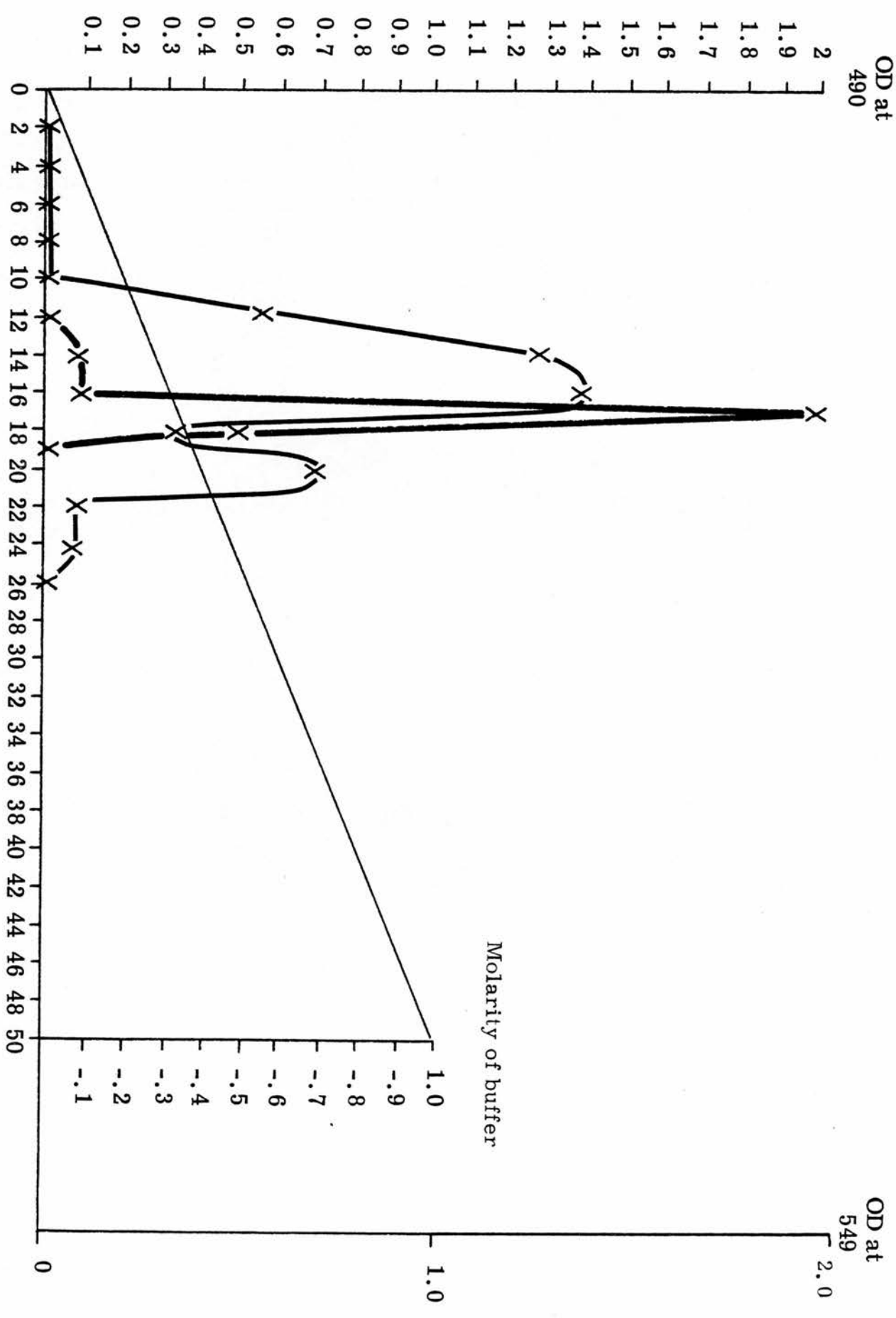

OD at 490
2
1.9
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
32
34
36
38
40
42
44
46
48
50
Molarity of buffer
1.0
-.9
-.8
-.7
-.6
-.5
-.4
-.3
-.2
-.1
0
1.0
OD at 549
2.0

Fig. 5.52 CIE of Ic fraction 1 with Ia type-specific antiserum.

Fig. 5.53 CIE of Ic fraction 2 with Ia type-specific antiserum.

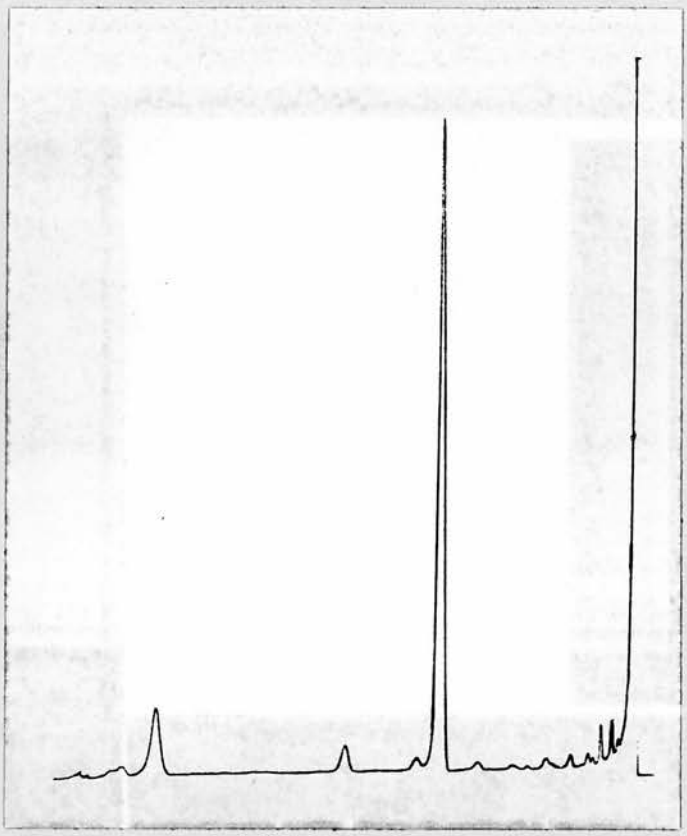


Fig. 5.54 GLC on column SP2330 of alditol acetate derivatives of the acid hydrolysed Ic fraction 1 indicating rhamnose, unknown sugar and galactose.

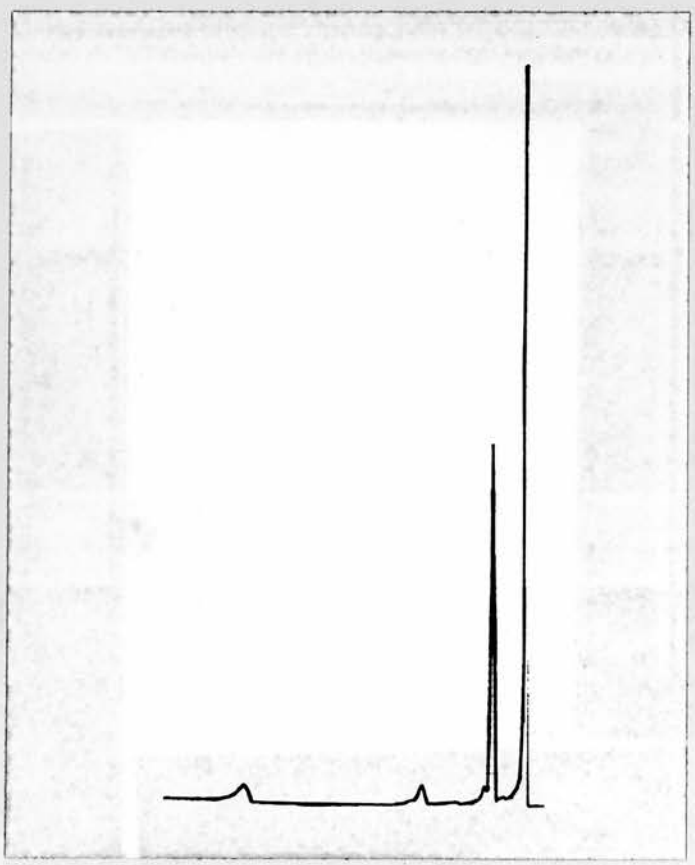


Fig. 5.55 GLC on column Ov225 of alditol acetate derivatives of the acid hydrolysed Ic fraction 1 indicating rhamnose, galactose and glucosamine.

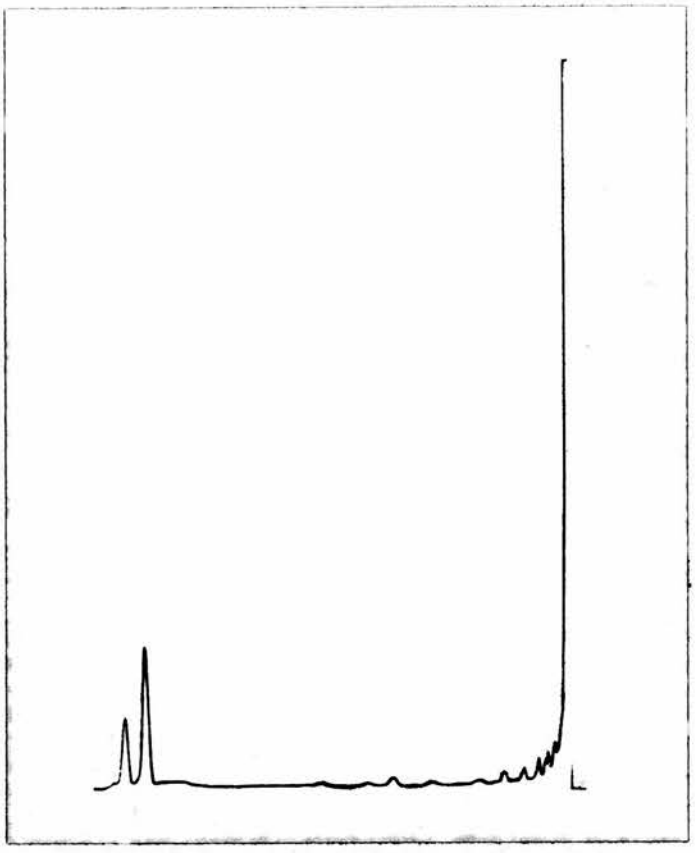


Fig. 5.56 GLC on column SP2330 of alditol acetate derivatives of the acid hydrolysed Ic fraction 2 indicating glucose and galactose.

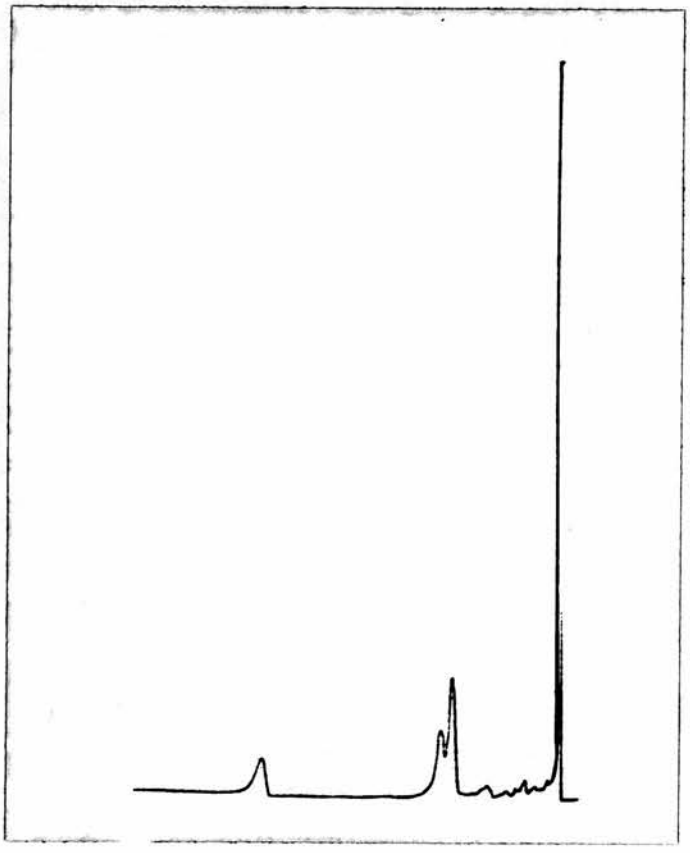


Fig. 5.57 GLC on column OV225 of alditol acetate derivatives of the acid hydrolysed Ic fraction 2 indicating galactose, glucose and glucosamine.

Fig. 5.58 CIE of "Lancefield-prepared" type Ic antigens with Ia type-specific antiserum. The precipitin lines are vii and viii.

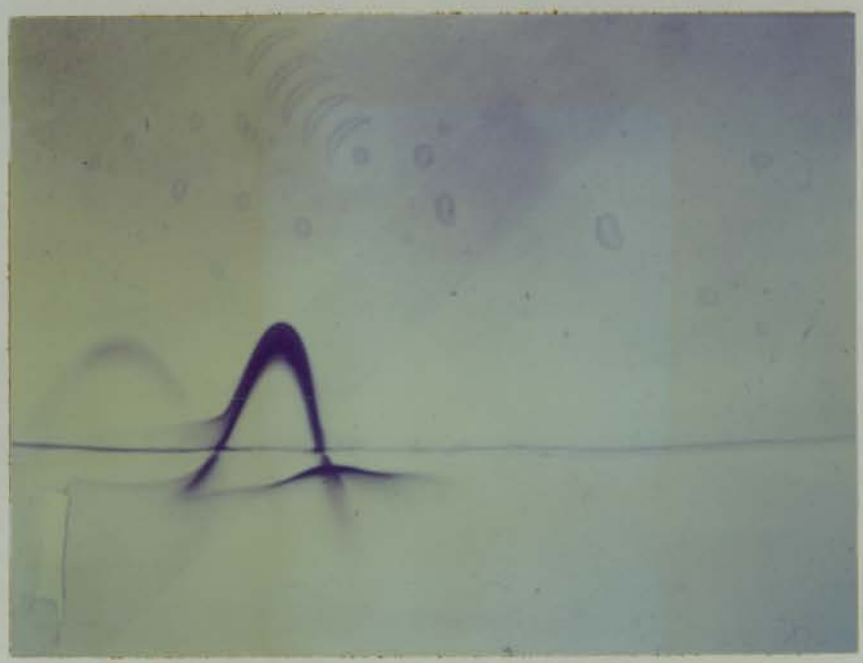


Fig. 5.59 CIE of "Lancefield-prepared" type Ic antigens with Ic type-specific antiserum. The precipitin lines are i, vii and viii.

Discussion

The main part of this study was concerned with the extraction and subsequent characterisation of antigenic carbohydrate complexes covalently attached to the cell walls of group B streptococcal serotypes Ia, Ib and Ic. Unlike many of the previous reports where antigens were extracted from untreated whole cells, in this study cell walls were collected and purified by SDS treatment, thereby removing all non-covalently bound polymers.

Release of carbohydrate from Ia cell walls was performed by three different hydrolysis procedures. Extraction using cold trichloroacetic acid resulted in the release of a significantly greater amount of carbohydrate, in comparison to the procedures using sodium hydroxide and hydrochloric acid. Purification of this extracted Ia carbohydrate was performed by ion-exchange chromatography and four distinct fractions were eluted from a DEAE-cellulose column by increasing molarities of pyridinium acetate buffer. The fact that these polymers were able to bind to DEAE-cellulose indicated that they possessed an overall negative charge.

The antigenic activity of the four fractions separated from the Ia cell wall carbohydrate with group B streptococcal antisera was visualised by performing crossed immunoelectrophoresis. Two of the fractions were shown to have no activity while the other two fractions both reacted with Ia type-specific antiserum. In addition, one of these fractions (2) also reacted with Ib antiserum but not Ic antiserum, while the other fraction (3) reacted with antiserum of type Ic but not Ib. It was considered notable that

neither of the fractions reacted with antiserum raised against whole cell preparations of serotypes II and III.

Chemical analysis of the antigenic fractions indicated that fraction 2 contained rhamnose, galactose, glucose and glucosamine in approximately equal proportions, and was essentially different from fraction 1 in possessing sialic acid. Fraction 3 was puzzling in that rhamnose appeared to be the sole carbohydrate constituent. It is probable that acid or heat labile components of this polymer were destroyed during the hydrolysis or derivitisation procedures. Kane and Karakawa (1977a) detected five carbohydrate fractions from whole cells of serotype Ia by heating the bacteria in phosphate-buffered saline and then separating the extract by DEAE-cellulose chromatography. Only one fraction, which possessed group-activity with group-specific antiserum, was considered to be associated with the peptidoglycan backbone of the cell, while the remaining fractions, which possessed type activity with homologous typing antiserum, were thought to originate from surface or capsular layers surrounding the cell wall. The chemical nature of the group-specific antigen from that study was similar to the components of fraction 2 in the present investigation, with the exception of glucose which was found in trace amounts only by Kane and Karakawa.

The purified carbohydrate extract from serotype Ib cell walls was shown by DEAE-cellulose chromatography to be negatively charged and consist of one distinct fraction only, containing galactose, glucosamine, glucose, rhamnose and sialic acid in the molar proportions 0.6:1.0:0.2:1.9:0.03 respectively. The constitution of this antigen was very similar to the group antigen isolated by

Kane and Karakawa (1977a) discussed previously.

Crossed immunoelectrophoresis of the Ib antigen with the five type-specific antisera indicated that it did not confer sub-type specificity within type I strains reacting with antisera of Ia, Ib and Ic serotypes, but displayed no activity with antisera of serotypes II and III.

Crossed immunoelectrophoresis studies of the extracted carbohydrate from Ic cell walls indicated that the antigenic profile of this extract was similar to that observed with the Ia cell wall antigens. However, chemical analysis of the two fractions separated by DEAE-cellulose showed that the Ic fractions were quiet distinct from the antigenic fractions obtained from bacteria of serotype Ia. A factor common to both sets of antigenic fractions of Ia and Ic carbohydrates was that in each case, the fractions which reacted with Ib antiserum contained sialic acid, whereas the fractions which reacted with Ic antiserum contained rhamnose but no sialic acid. Further studies whereby inhibition of the precipitin reactions between the separate components are necessary to prove that sialic acid in the case of the Ib reaction and rhamnose in the Ic reaction are indeed the determinant molecules.

The presence of trace amounts of phosphorus in each of the extracts from cell walls of serotypes Ia, Ib and Ic, and the trichloroacetic acid lability of the bond between the isolated carbohydrate and peptidoglycan suggest that a linkage unit containing phosphodiester bonds might be present. This would be analogous to the peptidoglycan-teichoic acid linkage found in other Gram-positive bacteria (Coley et al., 1978).

The two outstanding features of this investigation are: (1), the demonstration of two distinct antigenic secondary wall polymers covalently attached to the peptidoglycan component of the cell walls of group B streptococcal serotypes Ia and Ic. Although previous workers have suggested the existence of two or more determinants located on the same antigen within surface or type-specific carbohydrate complexes of group B streptococci, this is the first occasion that two separate cell wall antigens have been reported involving studies on beta-haemolytic streptococci; (2), that type I strains of group B streptococci possessed carbohydrate antigens, covalently linked to cell wall peptidoglycan as demonstrated by CIE. These antigens did not confer sub-type specificity (Ia, Ib, Ic) and antibodies to these antigens were not provoked by whole cell antigen preparations of serotypes II and III. It is, therefore, paradoxical that the 'group-specific' antigens of type I strains (i.e. the carbohydrate cell wall antigen) did not react with typing antisera to types II and III, although group B streptococci of types II and III reacted with commercially available group B antiserum. This evidence suggests that Lancefield's idea of a common cell wall group specific antigen throughout the five serotypes of group B streptococci is probably incorrect. Further studies are now necessary to examine the nature of the grouping antigens possessed by serotypes II and III.

The additional studies in this chapter using Lancefield prepared group and type-specific antigens in crossed immunoelectrophoresis indicated a degree of complexity of the complete antigenic profile of group B streptococci hitherto unrecognised. Since the

'Lancefield' type-specific antigens from whole cells were not present in the extracts obtained from purified cell walls, they can be described as capsular or possibly cytoplasmic membrane antigens not covalently bound to the cell wall. No attempt was made to characterise these antigenic complexes chemically but the nature of the electrophoresis technique enabled common antigens from the different preparations to be recognised. It was noted that many of the so called type-specific antigens in serotypes Ia, Ib and Ic were common to all three serotypes, thus explaining previous difficulties of specificity in serotyping by the enzyme-linked immunosorbent assay studies in Chapter 4, and the gel-slide diffusion techniques described in Chapter 3.

During preparation of this discussion Wagner et al. (1980) demonstrated by immunoelectron microscopy that the group-specific polysaccharide antigen of group B streptococci traverses the whole of the cell wall, and that type-specific antigens were only found on the cell wall outer surfaces. This is in complete agreement with the finding in this study that the group-specific polysaccharides are secondary wall polymers covalently bound to peptidoglycan.

In conclusion, the investigations presented in this chapter indicate that a serological identification system of increased specificity and greater reliability for group B streptococci can be developed when the remaining antigens have been characterised and their location on the cell surface determined. Of equal importance is the question of prophylaxis for patients thought to be at risk of group B streptococcal infection, especially pregnant women and neonates. Effective measures can only be successful if the vaccination preparation elicits meaningful protection against a range of antigens present in serotypes I, II and III group B streptococci.

Chapter 6

Conclusion

Conclusion

The investigations described in this thesis have included: (1) an assessment of efficient methods of sampling and identification procedures in the isolation of group B streptococci; (2) measurement of the prevalence and possible importance of group B streptococci in the upper respiratory tract of children; (3) development and testing of new techniques for serological differentiation of beta-haemolytic streptococci; and (4) the presentation of new evidence relating to the complex nature of antigens found within surface components of group B streptococcal cells.

(1) Sampling studies

The results of a laboratory-based trial to assess the recovery of streptococci on different swabs stored under various conditions, with and without transport media, indicated that, in general, buffered cotton wool swabs without transport media were equally as good as the best of the transport medium/swab kits. In view of reports mentioned in the literature advocating the use of transport media to prolong viability of bacteria stored on swabs, these results were surprising. However, additional studies recently undertaken (Ross and Cumming, in press) to assess the effects of transport media on survival of other respiratory tract pathogens (*Neisseria* spp., *Bordetella pertussis*, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *Haemophilus influenzae*) on swabs have confirmed the argument in favour of the use of plain swabs alone. The behaviour of certain Gram-negative bacilli may be different from the pathogens of the upper respiratory tract on swabs and transport media, however.

The degree of recovery of organisms from swabs held for very short periods of time was approximately 10% of the initial inoculum loaded onto the swab, i.e. 90% of bacteria were "lost" during transfer from the swab to the bacteriological growth plate. An efficiency rate such as this would suggest that the bacteriologist should exercise a great deal of caution in the interpretation of bacterial culture plates as an aid to clinical diagnosis of disease. Furthermore, it was unequivocally shown that accurate quantitative studies of bacterial status were impossible to perform from swab samples, whether or not transport media were used, since multiplication of organisms on the swab occurred frequently. It must be emphasised that clinical material such as pus or faeces will provide the bacteriologist with a more meaningful picture of current bacterial flora, providing these samples can be transferred to the laboratory without undue delay. It is recognised, however, that on many occasions collection of clinical material is impossible, and for many types of studies swabbing techniques are appropriate.

(2) Epidemiological studies

These were prompted by suggestions in the literature, from American workers especially, that the throat may be a significant reservoir and source of group B streptococci. A group of Edinburgh schoolchildren between the ages 5 to 18 years were randomly selected and sampled to ascertain prevalence of group B streptococci in the upper respiratory tract. The sampling technique for this investigation was considered to be of prime importance since the results from the previous chapter had shown that choice of swab could greatly affect results. It was decided to use a plain cotton wool swab and a swab/transport media kit which had previously produced the

best recovery of organisms in the laboratory trial. Statistical analysis of the results in the field study indicated that there was no significant difference between the two sampling devices in the isolation of beta-haemolytic streptococci. However, if only one series of swabs had been taken, a large number of streptococcal-positive individuals would have been missed.

The number of children carrying beta-haemolytic streptococci in the throat was almost 20%, but of these only 2% were group B streptococcal carriers. This figure broadly agrees with the results of other studies undertaken in Europe, but in comparison to reports of considerably higher carriage rates in children in the U.S.A., there is a real possibility that prevalence of group B streptococci in the throat is significantly different amongst the populations of the two continents.

(3) Serological studies

The procedures used for identification of beta-haemolytic streptococci were extremely reliable, with the exception of the bacitracin disk method to distinguish group A streptococci which can not be recommended. A new serological technique (Streptosec) was shown in this study to be a highly efficient, yet expensive, method of grouping streptococci. This particular test had the advantage over other well known commercial grouping preparations in that it allowed identification of organisms picked directly from the plate.

The attempts to adapt the enzyme-linked immunosorbent assay system (ELISA) as a reliable technique for rapid grouping of beta-haemolytic streptococci and typing of group B streptococci were

very time consuming and only partially successful. Once the problem of antigen binding to polystyrene had been overcome, and antisera were produced with the required specificity, the assay was seen to have considerable potential for grouping streptococci. Previous reports describing the ELISA technique for detection of antibody levels used incubation times for each of the stages totalling more than 24h. This amount of time was considered inappropriate for a rapid diagnostic test and therefore reagent concentrations and incubation times were adjusted to allow completion of the assay within 4h, i.e. the same time required by some of the commercial coagglutination methods for grouping streptococci. In subsequent comparative tests the developed ELISA procedure was shown to be completely reliable, simple to perform, and considerably less expensive than commercial preparations.

Experiments to adapt the ELISA procedure for typing of group B streptococci were unsuccessful. A number of alternative approaches in technique were attempted also with little success. The major problem encountered in this study was an inability to produce antisera of required specificity for use in ELISA. The development of monoclonal antibodies for serotypes of group B streptococci would almost certainly allow not only a successful application of serotyping of the organisms by ELISA, but would also provide a highly accurate method whereby, for example, neonatal antibody levels against the group B serotypes could be monitored. A technique such as this may enable much earlier recognition of high-risk subjects immediately after birth.

(4) Antigens of group B streptococci

The antigen complexes associated with the cell wall of group B streptococci were studied. One of the main reasons that prompted these studies were the obvious discrepancies in published reports relating to antigenic constituents of group B serotypes, as demonstrated by the ELISA studies. Hitherto, the majority of investigators have directed most attention to surface antigens extracted by mild acid or alkali treatment from whole cells. This type of preparation resulted in the release of a mixture of both group and type-specific antigens from unspecified sites within surface layers of the cells. A number of recent reports had indicated the importance of sialic acid as one of the primary determinant groups within the type-specific antigens of group B streptococci, but of late, little attention has been focused on the group carbohydrate antigen, or 'C substance' bound to the cell wall. In the present study, the aim was to obtain a purified component of the cell, viz, the cell wall and to determine the exact chemical nature and immunological role of the secondary wall polymer covalently bound to peptidoglycan. The adaptation of crossed immunoelectrophoresis techniques proved to be an extremely valuable method whereby immunological activity of antigens, with specific antisera, could be visualised.

The main outcome of this study indicated that the antigenic character of polymers bound to the cell wall was infinitely more complex that hitherto realised. The finding of two distinct secondary wall polymers in serotypes Ia and Ic is unique to the current knowledge of beta-haemolytic streptococcal cell surfaces.

In addition, sialic acid was shown to be the most likely determinant molecule for one of the antigen complexes. Of equal interest were the findings that, although the main antigenic carbohydrates isolated from the cell walls did not confer sub-type specificity within type I group B streptococci, no reaction between these antigens occurred with antisera raised against serotypes II and III. Thus, it can be surmised that a common cell wall, or group-specific antigen in the five serotypes of group B streptococci, such as described by Lancefield, does not exist.

The relevance of a thorough understanding of the character of group B streptococcal surface carbohydrates, which have the ability to elicit an immune response in an infected host is vital if a successful immunisation procedure is to be initiated amongst neonates considered to be 'high risk' cases. Further investigations are required to elucidate the character of cell wall antigens of group B streptococcal serotypes II and III, and in addition it is possible that unidentified lipid-containing complexes associated with the cell membrane may have a significant part to play.

Bibliography

ABDULLA, E.M. & SCHWAB, J.H. (1965). Immunological properties of bacterial cell wall mucopeptides. Proceedings of the Society of Experimental Biology and Medicine 118, 359.

ALEXANDER, J.G. (1952). Transport medium for gynaecological swabs. Journal of Obstetrics and Gynaecology 59, 246-248.

AMIES, C.R. & DOUGLAS, G. (1965) A modified formula for the preparation of Stuart's transport medium. Canadian Journal of Public Health 58, 296-300.

AMIES, C.R. (1967). A modified formula for the preparation of Stuart's transport medium. Canadian Journal of Public Health 58, 296-300.

AMINOFF, D. (1961). Methods for the quantitative estimation of N-acetylneuraminic acid and their application to hydrolysates of sialomucoids. Biochemical Journal 81, 384-392.

ANDERSON, A.J., ARCHIBALD, A.R. (1975). Poly(Glucosylglycerol Phosphate) teichoic acid in the walls of Bacillus stearothermophilus B65. Biochemical Journal 151, 115-120.

ANDERSON, K.F. (1965). Bacteriological swab. Letter to the Editor. British Medical Journal 2, 1123.

ANTHONY, B.F. & CONCEPCION, N.F. (1975). Group B streptococcus in a general hospital. Journal of Infectious Diseases 132, 561-567.

ANTHONY, B.F., OKADA, D. & HOBEL, C.J. (1975). Group B streptococci (GBS) in perinatal infections: natural history of maternal and neonatal colonisation (Abstract). Pediatric Research 9, 296.

ANTHONY, B.F., OKADA, D. & HOBEL, C.J. (1978). Epidemiology of group B streptococcus: longitudinal observations during pregnancy. Journal of Infectious Diseases 137, 524-528.

ARVILOMMI, H. (1976). Grouping of beta-haemolytic streptococci by using coagglutination, precipitation or bacitracin sensitivity. Acta Pathologica et Microbiologica Scandinavica Section B 84, 79-84.

AVRAMEUS, S. & URIEL, J. (1966). Methode de marquaze d'antigenes et d'anticorps avec des enzymes et son application en immunodiffusion. Compte rendu de l'Acadamie des Sciences Paris 262, 2543-2545.

AYERS, S.H. & RUPP, P. (1922). Differentiation of hemolytic streptococci from human and bovine sources by the hydrolysis of sodium hippurate. Journal of Infectious Diseases 30, 388-399.

BADRI, M.S., ZAWANEH, S., CRUZ, A.C., MANTILLA, G., BAER, H., SPELLACY, W.N. & AYOUB, E.M. (1977). Rectal colonization with group-B streptococcus: relation to vaginal colonisation in pregnant women. Journal of Infectious Diseases 135, 308-312.

BAILEY, W.R. & BYNOE, E.T. (1953). The "filter paper" method for collecting and transporting stools to the laboratory for enteric bacteriological examination. Canadian Journal of Public Health 44, 468-475.

BAKER, C.J., BARRETT, F.F. & GORDON, R.C. (1973). Suppurative meningitis due to streptococci of Lancefield group B: A study of 33 infants. Journal of Pediatrics 82, 724-731.

BAKER, C.J. & BARRETT, F.F. (1973). Transmission of group B streptococci among parturient women and their neonates. Journal of Pediatrics 83, 919-925.

BAKER, C.J., KASPER, D.L. & DAVIS, C.E. (1976). Immunochemical characterization of the "native" type III polysaccharide of group B streptococcus. Journal of Experimental Medicine 143, 258-270.

BAKER, C.J. (1977). Summary of workshop on perinatal infections due to group-B streptococci. Journal of Infectious Diseases 136, 137-152.

BARKULIS, S.S. & JONES, M.F. (1957). Studies of streptococcal cell walls. 1. Isolation, chemical composition and preparation of M protein. Journal of Bacteriology 74, 207-216.

BARTLETT, D.I. & HUGHES, M.H. (1969). Bacteriological swabs. British Medical Journal 3, 450-451.

BERGEY. p.157. In Bergey's Manual of Determinative Bacteriology by R.S. Breed, E.G.D. Murray and N.R. Smith, Seventh Edition, The Williams & Wilkins Company, Baltimore.

BERGQUIST, G. (1974). Neonatal infections caused by group B streptococci. III Incidence in Sweden 1970-71. Scandinavian Journal of Infectious Diseases 6, 29-31.

BEVANGER, L. & MAELAND, J.A. (1977). Type classification of group B streptococci by the fluorescent antibody test. Acta Pathologica et Microbiologica Scandinavica Section B 85, 357-362.

BLACK, W.A. & VAN BUSKIRK, F. (1973). Gentamicin blood agar used as a general-purpose selective medium. Applied Microbiology 25, 905-907.

BLANCHETTE, L.P. & LAWRENCE, C. (1967). Group A streptococcus screening with neomycin blood agar. American Journal of Clinical Pathology 37, 441-443.

BRAUNSTEIN, J., TUCKER, E.B. & GIBSON, B.C. (1969). Identification and significance of group B streptococci. American Journal of Clinical Pathology 51, 207-213.

THE BRITISH COMMUNICABLE DISEASE SURVEILLANCE CENTRE (1978). British Medical Journal 1, 55.

BROWN, J.H. (1919). The use of blood agar for the study of streptococci. Monograph of the Rockefeller Institute for Medical Research, No. 9, New York.

BROWN, J.H. (1920). The cultural differentiation of beta-haemolytic streptococci of human and bovine origin. Journal of Experimental Medicine 31, 35-47.

BROWN, J.H. (1937). Appearance of double-zone beta-hemolytic streptococci in blood agar. Journal of Bacteriology 34, 35-48.

BROWN, J.H. (1939). Double-zone beta-hemolytic streptococci: their cultural characteristics, serological grouping, occurrence and pathogenic significance. Journal of Bacteriology 37, 133-144.

BUTTER, M.N.W. & de MOOR, C.E. (1967). Streptococcus agalactiae as a cause of meningitis in the newborn, and of septicaemia in adults: differentiation of human and bovine varieties. Antonie van Leeunwenhoek Journal of Microbiology and Serology 33, 439-450.

CAIN, R.M. & STEELE, H. (1953). The use of calcium alginate soluble wool for the examination of cleansed eating utensils. Canadian Journal of Public Health 44, 464-467.

CAREY, R.B., EISENSTEIN, T.K., SHOCKMAN, G.D., GREBER, T.F. & SWENSON, R.M. (1980). Soluble group- and type-specific antigens from type III group B streptococcus. Infection and Immunity 28, 195-203.

CARLSSON, H.E., LINDBERG, A.A. & HAMMARSTROM, S. (1972). Titration of antibodies to salmonella O antigens by enzyme-linked immunosorbent assay. Infection and Immunity 6, 703-708.

CARS, O., FORSUM, U. & HJELM, E. (1975). New immunofluorescence method for the identification of group A, B, C, E, F and G streptococci. Acta Pathologica et Microbiologica Scandinavica Section B 83, 145-152.

CHEN, P.S., TORIBARA, T.Y. & WARNER, H. (1956). Microdetermination of phosphorus. Analytical Chemistry 28, 1756-1758.

CHRETIEN, J.H., McGINNISS, C.G., THOMPSON, J., DELAHA, E. & GARAGUSI, V.F. (1979). Group B beta-hemolytic streptococci causing pharyngitis. Journal of Clinical Microbiology 10, 263-266.

CHRISTENSEN, P., KAHLMETER, G., JONSSON, S. & KRONVALL, G. (1973). New method for the serological grouping of streptococci with specific antibodies adsorbed to protein A-containing staphylococci. Infection and Immunity 7, 881-885.

CHRISTENSEN, K.K. & CHRISTENSEN, P. (1979). Epidemiology of group B streptococcal carriage in the human throat and urogenital tract. In Pathogenic Streptococci, Reedbooks, edited by M.T. Parker pp. 182-183.

CHRISTENSEN, K.K., CHRISTENSEN, P., HOVELIUS, B., PETTERSSON, L., SCHALEN, C. & THIMANSSON, H. (1979). Upper respiratory tract spread of group B streptococci type Ib in a kindergarten. Scandinavian Journal of Infectious Diseases 11, 129-133.

CHRISTIE, R., ATKINS, N.E. & MUNCH-PETERSON, E. (1944). A note on a lytic phenomenon shown by group B streptococci. Australian Journal of Experimental Biology & Medical Science 22, 197-200.

CLARK, M.F. & ADAMS, A.N. (1977). Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology 34, 475-483.

CLOUTIER-LAMBIN, L. & GAUVREAU, L. (1968). Milieux selectifs a l'acide nalidixique et a l'alcool β-phenylethylique pour l'isolement de bacteries a Gram-positif. Revue de Canadian Biologie 27, 29-35.

COLEBROOK, L. & PURDIE, A.W. (1937). Treatment of 106 cases of puerperal fever by sulphanilamide (streptocide). Lancet 2, 1237.

COLEMAN, D.J., McGHIE, D. & TEBBUTT, G.M. (1977). Further studies on the reliability of the bacitracin inhibition test for the presumptive identification of Lancefield group A streptococci. Journal of Clinical Pathology 30, 421-426.

COLEY, J., TARELLI, E., ARCHIBALD, A.R. & BADDILEY, J. (1978). The linkage between teichoic acid and peptidoglycan in bacterial cell walls. FEBS Letters 88, 1-9.

COLLEE, J.G., WATT, B., BROWN, R. & JOHNSTON, S. (1974). The recovery of anaerobic bacteria from swabs. Journal of Hygiene, Cambridge 72, 339-347.

THE COMMISSION ON ACUTE RESPIRATORY DISEASES, U.S.A. (1947). The role of Lancefield groups of beta-hemolytic streptococci in respiratory infections. The New England Journal of Medicine 236, 157-166.

THE COMMISSION ON ACUTE RESPIRATORY DISEASES (1949). The single throat culture as an index of the bacterial flora of the respiratory tract. American Journal of Hygiene 50, 168-174.

COONS, A.H. & KAPLAN, M.H. (1950). Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. Journal of Experimental Medicine 91, 1-13.

COOPER, G.N. (1957). The prolonged survival of upper respiratory tract and intestinal pathogens on swabs. Journal of Clinical Pathology 10, 226-230.

COUNCILMAN, W.T. (1893). The pathology and diagnosis of diphtheria. American Journal of Medical Science 106, 540-552.

CROPP, C.B., ZIMMERMAN, R.A., JELINKOVA, J., AUERNHEIMER, A.H., BOLIN, R.A. & WYRICK, B.C. (1974). Serotyping of group B streptococci by slide agglutination, fluorescent microscopy and microimmunodiffusion. Journal of Laboratory and Clinical Medicine 84, 594-603.

CRUICKSHANK, R. (1953). Clinical pathology in general practice. Taking swabs. British Medical Journal 2, 1095-1097.

CUMMINS, C.S. & HARRIS, H. (1956). The chemical composition of the cell wall in some Gram-positive bacteria and its possible value as a taxonomic character. Journal of General Microbiology 14, 583-600.

CURTIS, S.N. & KRAUSE, R.M. (1964a). Identification of rhamnose as an antigenic determinant of group B streptococcal carbohydrate. Federation Proceedings 23, 191.

CURTIS, S.N. & KRAUSE, R.M. (1964b). Antigenic relationships between groups B and G streptococci. Journal of Experimental Medicine 120, 629-637.

DADD, A.H., DAGNALL, V.P., EVERALL, P.H. & JONES, A.C. (1970). The survival of streptococcus pyogenes on bacteriological swabs made from various fibres. Journal of Medical Microbiology 3, 561-572.

DAJANI, A.S. (1973). Rapid identification of beta-hemolytic streptococci by counterimmunoelectrophoresis. Journal of Immunology 110, 1702-1704.

DAMASK, L.J., MONTOYA, O. & AXELROD, J.L. (1979). Rapid slide agglutination test for Lancefield grouping of streptococci. Archives Pathology & Laboratory Medicine 103, 456-458.

DARLING, C.L. (1975). Standardization and evaluation of the CAMP reaction for the prompt, presumptive identification of Streptococcus agalactiae (Lancefield group B) in clinical material. Journal of Clinical Microbiology 1, 171-174.

DEELDER, A.M. (1975). Schistosoma mansoni: Immunohistoperoxidase procedure in Defined Antigen Substrate Spheres (DASS) system as serologic field test. Experimental Parasitology 37, 405-410.

DOWNIE, A.W. (1940). Survival of meningococci on swabs and blood agar. Lancet 2, 36-37.

DUBOIS, M., GILLIES, K.A., HAMILTON, J.K., REBERS, P.A. & SMITH, F. (1956). Colorimetric method for determination of sugars and related substances. Analytical Chemistry 28, 350-356.

DURAND, P. & GIRAUD, P. (1923). Les streptocoques chromogenes. Compte Rendu de l'Academie des Sciences, Paris clxxvii, 1333-1335.

EASMON, C., COX, S.E. & HOWARD, A. (1980). Grouping of beta-haemolytic streptococci by agglutination. Journal of Clinical Pathology 33, 386-389.

EDBERG, S.C. & SAMUELS, S. (1976). Rapid, colourimetric test for the determination of hippurate hydrolysis by group B streptococcus. Journal of Clinical Microbiology 3, 49-50.

EDERER, G.M., HERRMANN, M.M., BRUCE, R., MATSEN, J.M. & CHAPMAN, S.S. (1972). Rapid extraction method with Pronase B for grouping beta-hemolytic streptococci. Applied Microbiology 23, 285-288.

EDERER, G.M. & CHAPMAN, S. (1972). Simplified fluorescent-antibody staining method for primary plate isolation of Group A streptococci. Applied Microbiology 24, 160-161.

EDWARDS, E.A. & LARSON, G.L. (1974). New method of grouping beta-haemolytic streptococci directly on sheep-blood agar plates by coagglutination of specifically sensitized protein A-containing staphylococci. Applied Microbiology 28, 972-976.

EICKHOFF, T.C., KLEIN, J.O., DALY, A.K., INGALL, D. & FINLAND, M. (1964). Neonatal sepsis and other infections due to group B beta-hemolytic streptococci. The New England Journal of Medicine 271, 1221-1228.

EL GHOROURY, A.A. (1950). Comparative studies of group "B" streptococci of human and bovine origin. I. Cultural and biochemical characters. American Journal of Public Health 40, 1273-1277.

EL KHOLY, A., WANNAMAKER, L.W. & KRAUSE, R.M. (1974). Simplified extraction procedure for serological grouping of beta-hemolytic streptococci. Applied Microbiology 28, 836-839.

EL KHOLY, A., FACKLAM, R.R., SABRI, G. & ROTTA, J. (1978). Serological identification of group A streptococci from throat scrapings before culture. Journal of Clinical Microbiology 8, 725-728.

ELLNER, P.D. & ELLNER, C.J. (1966). Survival of bacteria on swabs. Journal of Bacteriology 91, 905-906.

ELSTON, H.R. (1965). Neomycin blood agar for the selective culture of pneumococcus and certain other bacteria. Journal of Pathology and Bacteriology 90, 336-338.

ENGVALL, E. & PERLMANN, P. (1971). Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G. Immunochemistry 8, 871-874.

ENGVALL, E. & PERLMANN, P. (1972). Enzyme-linked immunosorbent assay, ELISA. III. Quantitation of specific antibodies by enzyme-labelled anti-immunoglobulin in antigen-coated tubes. The Journal of Immunology 109, 129-135.

ENGVALL, E. & CARLSSON, H.E. (1976). In Immuno-enzymatic Techniques, Inserm, Paris.

ESSEVELD, H., DANIELS-BOSMAN, M.S. & LEIJNSE, B. (1958). Some observations about the CAMP reaction and its application to human beta-haemolytic streptococci. Leeuwenhoek Nederlands Tijdschr. 24, 145-156.

FACKLAM, R.R. (undated). Identification of group B streptococci. Centre for Disease Control, Atlanta, Georgia, U.S.A.

FALLON, R.J. (1975). The rapid identification of Lancefield group B streptococci. Journal of Clinical Pathology 27, 902-905.

FARRAR, J.L. & PAULL, A. (1979). Grouping of streptococci by means of latex particles coated with antibody. p.256 In Pathogenic Streptococci, ed. by M.T. Parker, Reedbooks.

FARRELL, B. & AMIRAK, I. (1976). Letter to the editor. Lancet 2, 1082.

FENTON, L.J. & HARPER, M.H. (1978). Direct use of counterimmunoelectrophoresis in detection of group B streptococci in specimens containing mixed flora. Journal of Clinical Microbiology 8, 500-502.

FERRIERI, P. & BLAIR, L.L. (1977). Pharyngeal carriage of group B streptococci: detection by three methods. Journal of Clinical Microbiology 6, 136-139.

FERRIERI, P., CLEARY, P.P. & SEEDS, A.E. (1977). Epidemiology of group B streptococcal carriage in pregnant women and newborn infants. Journal of Medical Microbiology 10, 103-114.

FINCH, R.G., FRENCH, G.L. & PHILLIPS, I. (1976). Group B streptococci in the female genital tract. British Medical Journal 2, 1245-1247.

FRANCIOSI, R.A., KNOSTMAN, J.D. & ZIMMERMAN, R.A. (1973). Group B streptococcal neonatal and infant infections. Journal of Pediatrics 82, 707-718.

FRANEK, J. & KUBIN, V. (1968). The use of fluorescent antibodies in the identification of streptococci. I. Identification of Streptococcus pyogenes group A. Journal of Hygiene, Epidemiology, Microbiology and Immunology 12, 82-90.

FRASER, A.G. (1975). Preparation of a glycoprotein fraction from pooled human plasma and its evaluation as a substrate for the assay of Clostridium welchii (C. perfringens) neuraminidase. Journal of Medical Microbiology 8, 235-249.

FREIMER, E.H. (1963). Studies of L forms and protoplasts of group A streptococci. II. Chemical and immunological properties of the cell membrane. Journal of Experimental Medicine 117, 377-379.

FREIMER, E.H. (1967). Type-specific polysaccharide antigens of group B streptococci. II. The chemical basis for serological specificity of the type II HCl antigen. Journal of Experimental Medicine 125, 381-392.

FRY, R.M. (1938). Fatal infections by haemolytic streptococcus group B. Lancet 1, 199-201.

FULLER, A.T. (1938). Formamide method for extraction of polysaccharides from haemolytic streptococci. British Journal of Experimental Pathology 19, 130-139.

GHUYSEN, J.M. (1968). Use of bacteriolytic enzymes in determination of wall structures and their role in cell metabolism. Bacteriology Revue 32, 425-464.

GOLDSCHEIDER, A. (1893). Diagnostik der nerventrankheiten. Zentralblatt fur Klinical Medicine 22, 534.

GRAY, B.M. (1979). ELISA methodology for polysaccharide antigens: protein coupling of polysaccharides for adsorption to plastic tubes. Journal of Immunological Methods 28, 187-192.

GREER, D.O., POLLOCK, D.A. & PEARSON, J.K.L. (1978). A comparison between four serological methods used in the identification of Streptococcus agalactiae. British Veterinary Journal 134, 572-577.

HAMILTON, D.J. (1895). A ready means of procuring and transmitting diphtheritic discharges for examination. British Medical Journal 1, 298.

HAMILTON, W.J. (1972). Streptococcal grouping: heat extraction of C substance from beta-haemolytic streptococci. Medical Laboratory Technology 29, 385-388.

HARDMAN, W. (1895). Another ready means of transmitting diphtherial and other discharges. British Medical Journal 1, 387.

HARE, R. (1935). The classification of haemolytic streptococci from the nose and throat of normal human beings by means of precipitin and biochemical tests. Journal of Pathology and Bacteriology 41, 499-512.

HARPER, I.A. (1971). The importance of group B streptococci as human pathogens in the British Isles. Journal of Clinical Pathology 24, 438-441.

HAUG, R.H. (1972). Type classification of the group B streptococci by means of Lancefield's precipitin method. Classification of strains of bovine and human origin isolated during the year 1971 in Norway. Nordiske Veterinaer Medicin 24, 631-638.

HAUG, R.H. & SØDERLUND, E. (1977). Brief Report. Pigment production in group B streptococci. Acta pathologica et microbiologica scandinavica Section B 85, 286-288.

HEWLETT, R.T. & NOLAN, H. (1896). Results of the bacteriological examination of 1,000 cases of suspected diphtheria. British Medical Journal 1, 266.

HIGGINS, M. & MARCELLA, A. (1950). In Public Health Laboratory Service Bulletin (Great Britain), February 1950, 50-51.

HILL, A.M. & BUTLER, H.M. (1940). Haemolytic streptococcal infections following childbirth and abortion : clinical features, with special reference to infections due to streptococci of groups other than A. Medical Journal of Australia 1, 293-299.

HILL, H.R., RITER, M.E., MENGE, S.K., JOHNSON, D.R. & MATSEN, J.M. (1975). Rapid identification of group B streptococci by counterimmunoelectrophoresis. Journal of Clinical Microbiology 1, 188-191.

HITCHCOCK, C.H. (1924). Classification of the hemolytic streptococci by the precipitin reaction. Journal of Experimental Medicine 40, 445-452.

HOLLINGER, N.F. & LINDBERG, L.H. (1958). Delayed recovery of streptococci from throat swabs. American Journal of Public Health 48, 1162-1169.

HOLLINGER, N.F. & RANTZ, L. (1959). In pursuit of the streptococcus: newer techniques for their recovery and identification, and clinical implications. Pediatrics 24, 1112-1117.

HOLMGREN, J. & SVENNERHOLM, A.M. (1973). Enzyme-linked immunosorbent assays for cholera serology. Infection and Immunity 7 759-763.

HOOD, M., JANNEY, A. & DAMERON, G. (1961). Beta-hemolytic streptococcus group B associated with problems of perinatal period. American Journal of Obstetrics and Gynaecology 82, 809-818.

HOSTY, T.S., JOHNSON, M.B., FREEAR, M.A., GADDY, R.E. & HUNTER, F.R. (1964). Evaluation of the efficiency of four different types of swabs in the recovery of group A streptococci. Health Laboratory Sciences 1, 163-169.

HOWARD, J.B. & McCRACKEN, G.H. (1974). The spectrum of group B streptococcal infections in infancy. American Journal of Diseases of Children 128, 815-818.

HWANG, M.N. & EDERER, G.M. (1975). Rapid hippurate hydrolysis method for presumptive identification of group B streptococci. Journal of Clinical Microbiology 1, 114-115.

THE INTERNATIONAL CONGRESS FOR MICROBIOLOGY, U.S.A. (1936).

ISLAM, A.K. (1977). Rapid recognition of group B streptococci. Lancet Jan. 29th 256-257.

JELINKOVA, J., NEUBAUER, M. & DUBEN, J. (1970). Group B streptococci in human pathology. Zentralblatt fur Bacteriologie, Parasitenkunde, Infektionskrankbeiten und Hygiene 214, 450-457.

JELINKOVA, J. (1977). Group B streptococcal infections. Current Topics in Microbiology and Immunology 76, 127-131.

JODAL, U., AHLSTEDT, S., CARLSSON, B., HANSON, L.A., LINDBERG, U. & SOHL, A. (1974). Local antibodies in childhood urinary tract infection. International Archives of Allergy and Applied Immunology 47, 537-546.

JOE, L.K. (1950). A simple method of sending dysenteric faeces to a laboratory. Documenta Neerlandica et Indonesica de morbis Tropicis 2, 97.

JOE, L.K. (1956). The dried filter paper technique for sending stool specimens to a laboratory for bacteriologic examination. American Journal of Tropical Medicine 5, 133-135.

JOKIPII, A. & JOKIPII, S. (1976). Presumptive identification and antibiotic susceptibility of group B streptococci. Journal of Clinical Pathology 29, 736-739.

JONES, H.E. & HOWELLS, C.H.L. (1968). Neonatal meningitis due to Streptococcus agalactiae. Postgraduate Medical Journal 44, 549-551.

KANE, J.A. & KARAKAWA, W.W. (1977a). Multiple polysaccharide antigens of group B streptococci, type Ia: emphasis on a sialic acid-containing type-specific polysaccharide. Journal of Immunology 117, 2155-2160.

KANE, J.A. & KARAKAWA, W.W. (1977b). Existence of multiple immunodeterminants in the type-specific capsular substance of group B type Ia streptococci. Infection and Immunity 19, 983-991.

KARAKAWA, W.W., KRAUSE, R.M. & BORMAN, E.K. (1965). Immunochemical aspects of the cross-reactivity between groups A and C streptococci as detected by the fluorescent antibody technique. Journal of Immunology 94, 282-288.

KARAKAWA, W.W. & KRAUSE, R.M. (1966). Studies on the immunochemistry of streptococcal mucopeptide. Journal of Experimental Medicine 124, 155-171.

KARAKAWA, W.W., LACKLAND, H. & KRAUSE, R.M. (1967). Antigenic properties of the hexosamine polymer of streptococcal mucopeptide. Journal of Immunology 99, 1178-1182.

KEITEL, H.G., HANANIAN, J., TING, R., PRICE, L.N. & RANDELL, E. (1962). Meningitis in the newborn infant: report of 3 cases in whom the same organisms (pneumococci and group B streptococci) were recovered from the spinal fluid and maternal cervix. Journal of Pediatrics 61, 39-45.

KENNY, J.F. & ZEDD, A.J. (1977). Recurrent group B streptococcal disease in an infant associated with ingestion of infected mother's milk. Journal of Pediatrics 91, 158-159.

KEXEL, G. & BECK, K.J. (1965). Untersuchen uber die haufigkeit der B-streptokokken in Wochenbett. Geburtshilfe und Frauenheilkunde 25, 1078-1085.

KIDSON, A. (1967). A new selective medium for Streptococcus pyogenes and other streptococci. Journal of Medical Laboratory Technology 24, 179-186.

KJEMS, E. & HENRICHSEN, J. (1979). New serotypes of group B streptococci isolated from human sources. Journal of Clinical Microbiology 10, 109-110.

KJEMS, E., PERCH, B. & HENRICHSEN, J. (1979). Incidence of serious neonatal infections due to group B streptococci in Denmark. In Pathogenic Streptococci, Reedbooks, ed. by M.T. Parker pp. 173-174.

KNOX, J.M. (1979). Group B streptococcal infection. British Journal of Venereal Diseases 55, 118-120.

KOLLE, W. & WASSERMANN, A. (1903). In Handbuch der pathogenen Mikroorganismen, Vol. 3, Fischer, Jena.

KOSHI, G., THANGAVELU, C.P. & BRAHMADATHAN, K.N. (1979). The reliability and rapidity of the coagglutination technic and its comparison with precipitin technics in the grouping of streptococci. American Journal of Clinical Pathology 71, 709-712.

KRAUSE, R.M. & McCARTY, M. (1961). Studies on the chemical structure of the streptococcal cell wall. I. The identification of a mucopeptide in the cell walls of groups A and A-variant streptococci. Journal of Experimental Medicine 114, 127-140.

KRONVALL, G. & FROMMEL, D. (1970). Definition of staphylococcal protein A reactivity for human immunoglobulin G fragments. Immunochemistry 7, 124-129.

KRONVALL, G. (1973). A surface component in group A, C and G streptococci with non-immune reactivity for immunoglobulin G. Journal of Immunology 111, 1401-1406.

KUBIN, V., FRANEK, J. & PROCHAZKA, O. (1968). Use of fluorescent antibodies for the identification of streptococci. II. Determination of Streptococcus agalactiae (Group B). Journal of Hygiene, Epidemiology, Microbiology and Immunology 12, 315-323.

KUNTER, E. (1965). Gewinnung des prazipitierenden gruppenspezifischen Streptokokken-Polysaccharids durch Erlutzen von Streptokokken im Autoklaven. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionstrantiheiten und Hygiene 197, 72-78.

LANCASTER, L.J. & SHERRIS, J.C. (1960). An agar-diffusion grouping technic for beta hemolytic streptococci. American Journal of Clinical Pathology 34, 131-132.

LANCEFIELD, R.C. (1928). Antigenic complex of Streptococcus haemolyticus: demonstration of type-specific substance in extracts of Streptococcus haemolyticus. Journal of Experimental Medicine 47, 91-103.

LANCEFIELD, R.C. (1933). A serological differentiation of human and other groups of hemolytic streptococci. Journal of Experimental Medicine 57, 571-595.

LANCEFIELD, R.C. (1934). Serological differentiation of specific types of bovine hemolytic streptococci (group B). Journal of Experimental Medicine 59, 441-458.

LANCEFIELD, R.C. (1938). Two serological types of group B hemolytic streptococci with related, but not identical, type specific substances. Journal of Experimental Medicine 67, 25-40.

LANCEFIELD, R.C. & FREIMER, E.H. (1966). Type-specific polysaccharide antigens of group B streptococci. Journal of Hygiene 64, 191-203.

LANCEFIELD, R.C., McCARTY, M. & EVERLY, W.N. (1975). Multiple mouse-protective antibodies directed against group B streptococci. Special reference to antibodies effective against protein antigens. Journal of Experimental Medicine 142, 165-176.

LE MINOR, L., LE MINOR, S. & COMBES, R. (1949). Valeur du milieu de Moffet, Young et Stuart pour le transport des prelevements en vue de la culture du gonocoque. Annuals of the Institute Pasteur 77, 327-329.

LESHER, G.Y., FROELICH, E.J., GRUETT, M.D., BAILEY, J.H. & BRUNDAGE, R.P. (1962). 1,8-Naphthyridine derivitives. A new class of chemotherapeutic agents. Journal of Medical Pharmacology and Chemistry 5, 1063-1065.

LILLEY, B.D. & BREWER, J.H. (1953). Selective antibacterial action phenyethyl alcohol. Journal of the American Pharmaceutical Association 42, 6-8.

LIM, D.V., SMITH, R.D. & DAY, S. (1979). Evaluation of an improved rapid coagglutination method for the serological grouping of beta-hemolytic streptococci. Canadian Journal of Microbiology 25, 40-43.

LOWBURY, E.J. & LILLY, H.A. (1955). Selective plate medium for Cl. welchii. Journal of Pathology and Bacteriology 70, 105-109.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. Journal of Biological Chemistry 193, 265-275.

LUCAS, A. & ROBERTS, C.D. (1978). Group B streptococci in pooled human milk. British Medical Journal 2, 919-921.

MAHER, E. & IRWIN, R.C. (1966). Group B streptococcal infection in infancy: a case report and review. Pediatrics 38, 659-660.

MAIR, M.S. & McSWIGGAN, D.A. (1965). Antibacterial bacteriological swab (Letter). British Medical Journal 2, 230.

MARTIN, D.R., STANHOPE, J.M. & FINCH, L.A. (1977). Delayed culture of group A streptococci: an evaluation of variables in methods of examining throat swabs. Journal of Medical Microbiology 10, 249-253.

MAURER, M., THIRUMOORTHI, M.C. & DAJANI, A.S. (1979). Group B streptococcal colonization in prepubertal children. Pediatrics 64, 65-67.

MAXTED, W.R. (1948). Preparation of streptococcal extracts for Lancefield grouping. Lancet 2, 255-256.

MAXTED, W.R. (1949). Occurrence of the M substance of type 28 group A in streptococci of Lancefield groups B, C and G. Journal of General Microbiology 3, 1-6.

MAXTED, W.R. (1953). The use of bacitracin for identifying group A haemolytic streptococci. Journal of Clinical Pathology 6, 224-226.

McBRIDE, W.H. (1979). Dept. of Bacteriology, University of Edinburgh Medical School. Personal communication.

MERRITT, K. & JACOBS, N.J. (1976). Improved medium for detecting pigment production by group B streptococci. Journal of Clinical Microbiology 4, 379-380.

MOODY, M.D., ELLIS, E.C. & UPDYKE, E.L. (1958). Staining bacterial smears with fluorescent antibody. IV. Grouping streptococci with fluorescent antibody. Journal of Bacteriology 75, 553-560.

NAKANE, P.K. & PIERCE, G.B. (1966). Enzyme-labelled antibodies: preparation and application for the localization of antigens. Journal of Histochemistry and Cytochemistry 14, 929-931.

NAKANE, P.K. (1975). Localisation of hormones with the peroxidase-labelled antibody method. Methods in Enzymology 37, Part B, 133-144.

NICHOLAS, W.C. & STEELE, C.P. (1962). Occurrence of groupable beta-hemolytic streptococci. The Journal of the American Medical Association 181, 197-205.

NOBLE, R.C. & PENNY, B.B. (1974). A comparison by gel diffusion of the Lancefield and Rantz extraction techniques used in grouping haemolytic streptococci. Medical Laboratory Technology 31, 43-49.

NOCARD, M. & MOLLEREAU, G. (1887). Sur une mammite contagieuse des vaches laitieres. Annals of the Institute Pasteur 1, 109-126.

NOMOTO, M. & NAROHASHI, Y. (1959). A proteolytic enzyme of Streptomyces griseus. IV. General properties of Streptomyces griseus protease. Journal of Biochemistry 46, 1645-1651.

NYHAN, W.L. & FOUSEK, M.D. (1958). Septicemia of newborn. Pediatrics 22, 268-278.

ORLA-JENSEN, P. (1919). p.116 In The Lactic Acid Bacteria, Høst, Copenhagen.

OUCHTERLONY, O. (1949). In vitro method for testing the toxin-producing capacity of diphtheria bacteria. Acta pathologica et microbiologica scandinavica 25, 186.

PAREDES, A., WONG, P., MASON, E.O., TABER, L.H. & BARRETT, F.F. (1977). Nosocomial transmission of group B streptococci in a newborn nursery. Pediatrics 59, 679-682.

PARKER, M.T. & BALL, L.C. (1976). Streptococci and aerococci associated with systemic infection in man. Journal of Medical Microbiology 9, 275-302.

PARKER, M.T. (1977). Neonatal streptococcal infections. Postgraduate Medical Journal 53, 598-606.

PARKER, M.T. (1979). Infections with group-B streptococci. Journal of Antimicrobial Chemotherapy 5 (Suppl. A), 27-37.

PARKER, M.T. & STRINGER, J. (1979). The pattern of systemic disease due to group B streptococci. In Pathogenic Streptococci, Reedbooks, edited by M.T. Parker pp. 171-172.

PASS, M.A., GRAY, B.M., KHARE, S. & DILLON, H.C. (1979). Prospective studies of group B streptococcal infections in infants. Journal of Pediatrics 95, 437-443.

PATTISON, I.H., MATTHEWS, P.R., MAXTED, W.R. (1955a). Type classification by Lancefield's precipitin method of human and bovine group-B streptococci isolated in Britain. Journal of Pathology and Bacteriology 69, 43-50.

PATTISON, I.H., MATTHEWS, P.R. & HOWELL, D.G. (1955b). The type classification of group-B streptococci, with special reference to bovine strains apparently lacking in type polysaccharide. Journal of Pathology and Bacteriology 69, 51-60.

PEARSON, E.S. & HARTLEY, H.O. (1954). Table 8, in Biometrikia Tables for Statisticians, Vol. 1, Cambridge University Press.

PETRAN, E.J. (1964). Comparison of the fluorescent antibody and the bacitracin disk methods for the identification of group A streptococci. American Journal of Clinical Pathology 41, 224-226.

PIKE, R.M. (1945). The isolation of hemolytic streptococci from throat swabs. Experiments with sodium azide and crystal violet in enrichment broth. American Journal of Hygiene 41, 211-220.

PLUMMER, H. (1941). A serological and biochemical study of hemolytic streptococci. Journal of Immunology 42, 91-97.

POLLOCK, H.M. & DAHLGREN, B.J. (1973). Distribution of streptococcal groups in clinical specimens with evaluation of bacitracin screening. Applied Microbiology 27, 141-143.

POLLOCK, M.R. (1948). Unsaturated fatty acids in cotton wool plugs. Nature 4100, 853.

POXTON, I.R. (1979). Serological identification of Bacteroides species by an enzyme-linked immunosorbent assay. Journal of Clinical Pathology 32, 294-298.

PRESTON, W.J.D. (1896). The value of bacteriological examination in throat infections suggesting diphtheria. British Medical Journal 1, 1243.

QUIRANTE, J. & CASSADY, G. (1972). Group B beta-hemolytic streptococcal sepsis in the newborn. Clinical Research 20, 104.

RANTZ, L.A. & RANDALL, E. (1955). Use of autoclaved extracts of hemolytic streptococci for serological grouping. Stanford Medical Bulletin 13, 290-291.

REDYS, J.J., HIBBARD, E.W. & BORMAN, E.K. (1968). Improved dry-swab transportation for streptococcal specimens. Public Health Reports 83, 143-149.

REID, T.M.S. (1975). Emergence of group B streptococci in obstetric and perinatal infections. British Medical Journal 2, 533-538.

RISSING, J.P., BUXTON, T.B., MOORE, W.L., OZAWA, T. & MOORE, W.L. (1978). Enzyme-linked immunospecific antibody test for detecting antibody to Klebsiella. Journal of Clinical Microbiology 8, 704-707.

ROGERS, K.B. (1970). Neonatal meningitis and pneumonia due to Lancefield group B streptococci. Archives of Diseases Children 45, 147.

ROMERO, R. & WILKINSON, W. (1974). Identification of Group B Streptococci by immunofluorescence staining. Applied Microbiology 28, 199-204.

ROSENDAL, K. (1956). Grouping of hemolytic streptococci belonging to groups A, C and G: a comparison between the results obtained by precipitation and by slide-agglutination. Acta Pathologica et Microbiologica Scandinavica 39, 127-136.

ROSNER, R. (1977). Laboratory evaluation of a rapid four-hour serological grouping of groups A, B, C and G beta-streptococci by the Phadebact streptococcus test. Journal of Clinical Microbiology 6, 23-26.

ROSS, P.W. (1971). Throat swabs and swabbing technique. The Practitioner 207, 791-796.

ROSS, P.W. (1977). The isolation of Streptococcus pyogenes from throat swabs. Journal of Medical Microbiology 10, 69-76.

ROSS, P.W. (1980). Group B streptococci in women attending a sexually transmitted disease clinic. Journal of Infection, in press.

ROSS, P.W. & LOUGH, H. (1978). Survival of upper respiratory tract bacteria on cotton-wool swabs. Journal of Clinical Pathology 31, 430-433.

ROTE, N.S., TAYLOR, N.L., SHIGEOKA, A.O., SCOTT, J.R. & HILL, H.R. (1980). Enzyme-linked immunosorbent assay for group B streptococcal antibodies. Infection and Immunity 27, 118-123.

ROTTA, J., PRENDERGAST, T.J., KARAKAWA, W.W., HARMON, C.K. & KRAUSE, R.M. (1965). Enhanced resistance to streptococcal infection induced in mice by cell-wall mucopeptide. Journal of Experimental Medicine 122, 877-885.

ROUX, E. & YERSIN, A. (1890). Contribution a l'etude de la diphterie. Annals de l'Institute Pasteur 4, 385-426.

RUBBO, S.D. & BENJAMIN, M. (1951). Some observations on survival of pathogenic bacteria on cotton-wool swabs: development of a new type of swab. British Medical Journal 1, 983-987.

RUSSELL, H. & NORCROSS, N.L. (1972). The isolation and some physiochemical and biologic properties of the type III antigen of group B streptococci. Journal of Immunology 109, 90-96.

RUSSELL, H., FACKLAM, R.R. & EDWARDS, L.R. (1976). Enzyme-linked immunosorbent assay for streptococcal M protein antibodies. Journal of Clinical Microbiology 3, 501-505.

SASLAW, M.S. & JABLON, J.M. (1960). Epidemiology of group A beta-haemolytic streptococci as related to acute rheumatic fever in Miami, Fla.: Six-year study. Circulation 21, 679-683.

SCHAUF, V. & HLAING, V. (1976). Group B streptococcal colonization in pregnancy. Obstetrics and Gynaecology 47, 719-921.

SCHREINER, R.L., COATES, T. & SHACKLEFORD, P.G. (1977). Possible breast-milk transmission of group B streptococcal infection. Journal of Pediatrics 91, 159.

SHUKLA, R.N. & GUPTA, S.P. (1965). Beta-haemolytic streptococci: Comparative study of the methods of extraction of group specific antigen substance (polysaccharide 'C substance') of beta-haemolytic streptococci for grouping. Indian Journal of Medical Research 53, 962-964.

SLACK, M.P.E. & MAYON-WHITE, R.T. (1978). Group B streptococci in pharyngeal aspirates at birth and the early detection of neonatal sepsis. Archives of Disease in Childhood 53, 540-544.

SLADE, H.D. & SLAMP, W.C. (1962). Cell-wall composition and the grouping antigens of streptococci. Journal of Bacteriology 84, 345-351.

SMITH, R.E., PEASE, N.M.F., REIQUAM, C.W. & BEATTY, E.C. (1965). A comparison of multiple technics in the recovery of group A streptococci from throat cultures of children. The American Journal of Clinical Pathology 44, 689-694.

SMITH, R. (1971). Clinical application of immunofluorescence. III. Identification of Lancefield Group B Streptococci. Infection and Immunity 4, 189-193.

STABLEFORTH, A.W. (1932). Studies on bovine mastitis. VII. The serological characters of mastitis streptococci. Journal of Comparative Pathology and Therapeutics 45, 185-194.

STABLEFORTH, A.W. (1937). Serological types of Str. agalactiae (Streptococcus group B) in this and other countries. Journal of Pathology and Bacteriology 45, 263-277.

STABLEFORTH, A.W. (1946). p.203 In Little and Plastridge's Bovine mastitis, London.

STARK, C.N. & HERRINGTON, B.L. (1931). The drying of bacteria and the viability of dry bacterial cells. Society of American Bacteriologists. Thirty-second annual meeting, 13 (abstract).

STEERE, A.C., ABER, R.C., WARFORD, L.R., MURPHY, K.E., FEELEY, J.C., HAYES, P.S., WILKINSON, H.W. & FACKLAM, R.R. (1975). Possible nosocomical transmission of group B streptococci in a newborn nursery. Journal of Pediatrics 87, 784-787.

STETSON, C. (1965). p.304 In Bacterial and Mycotic Infections of Man, ed. by R.J. Dubos and J.G. Hirsch, J.B. Lippincott Company, Philadelphia.

STONER, R.A. (1978). Bacitracin and coagglutination for grouping of beta-hemolytic streptococci. Journal of Clinical Microbiology 7, 463-466.

STUART, R.D. (1946). Diagnosis and control of gonorrhoea by bacteriological cultures, with preliminary report on new method for transporting clinical material. Glasgow Medical Journal 27, 131-142.

STUART, R.D.,TOSHACH, S.R. & PATSULA, T.M. (1954). The problem of transport of specimens for culture of Gonococci. Canadian Journal of Public Health 45, 73-83.

STUART, R.D. (1956). Transport problems in public health bacteriology. Canadian Journal of Public Health 47, 114-122.

SWIFT, H.F., WILSON, A.T. & LANCEFIELD, R.C. (1943). Typing group A hemolytic streptococci by M-precipitin reactions in capillary pipettes. Journal of Experimental Medicine 78, 127-132.

TAI, J.Y., GOTSCHLICH, E.C. & LANCEFIELD, R.C. (1979). Isolation of type-specific polysaccharide antigen from group B type Ib streptococci. Journal of Experimental Medicine 149, 58-66.

TAPLIN, D. & LANSDELL, L. (1973). Value of desiccated swabs for streptococcal epidemiology in the field. Applied Microbiology 25, 135-138.

TREVELYAN, W.E., PROCTER, D.P. & HARRISON, J.S. (1950). Detection of sugars on paper chromatograms. Nature (London) 166, 444-445.

TRUANT, J.P., HADLEY, I.K. & BOYD, T.T. (1965). A comparison of the immunofluorescence technique with conventional methods for the identification of group A beta-haemolytic streptococci. Henry Ford Hospital Medical Bulletin 13, 357-360.

TUOMI, J. & NURMI, E. (1964). Identification of Streptococcus agalactiae by the fluorescent antibody. Acta veterinaar scandinavica 5, 50-55.

UPDYKE, E.L. (1956). Laboratory problems in the diagnosis of streptococcal infections. Laboratory Science Abstracts 47, 615.

VAN WEEMEN, B.K. & SCHUURS, A.H. (1971). Immunoassay using antigen-enzyme conjugates. FEBS Letters 15, 232-235.

VAN WEEMEN, B.K. & SCHUURS, A.H. (1972). Immunoassay using hapten-enzyme conjugates. FEBS Letters 24, 77-81.

VAN REIMSDIJK, M. (1924). Untitled. Zentralblatt fur Hygiene und Infectkr. 103, 106.

VINCENT, W.F., GIBBONS, W.E. & GAAFAR, H.A. (1971). Selective medium for the isolation of streptococci from clinical specimens. Applied Microbiology 22, 942-943.

VOLLER, A., BIDWELL, D., HULDT, G. & ENGVALL, E. (1974). A microplate method of enzyme-linked immunosorbent assay and its application to malaria. Bulletin of the World Health Organisation 51, 209-211.

VOLLER, A., BIDWELL, D.E. & BARTLETT, A. (1975). Microplate enzyme immunoassays for the immunodiagnosis of virus infections, p.506-512. In N.R. Rose and H. Friedman (Ed.), Manual of Clinical Immunology, American Society for Microbiology, Washington, D.C.

VOLLER, A., BARTLETT, A., BIDWELL, D.E., CLARK, M.F. & ADAMS, A.N. (1976). The detection of viruses by enzyme-linked immunosorbent assay (ELISA). Journal of General Virology 33, 165-167.

WADSTROM, T., NORD, C-E, LINDBERG, A.A. & MOLLBY, R. (1974). Rapid grouping of streptococci by immunoelectrophoresis. Medical Microbiology and Immunology 159, 191-200.

WAGNER, B., WAGNER, M., KUBIN, V. & RYC, M. (1980). Immunoelectron microscopic study of the location of group-specific and protein type-specific antigens of group B streptococci. Journal of General Microbiology 118, 95-105.

WATSON, B.K., MOELLERING, R.C. & KUNZ, L.J. (1975). Identification of streptococci: use of lysozyme and Streptomyces albus filtrate in the preparation of extracts for Lancefield grouping. Journal of Clinical Microbiology 1, 274-278.

WEBB, B.J., EDWARDS, M.S. & BAKER, C.J. (1980). Comparison of slide coagglutination test and countercurrent immunoelectrophoresis for detection of group B streptococcal antigen in cerebrospinal fluid from infants with meningitis. Journal of Clinical Microbiology 11, 263-265.

WEEKE, B. (1973a). General remarks on principles, equipment, reagents and procedures. Scandinavian Journal of Immunology 2, Supplement 1, 15-35.

WEEKE, B. (1973b). Crossed immunoelectrophoresis. Scandinavian Journal of Immunology 2, Supplement 1, 47-56.

WHITE, W.D. (1965). Antibacterial bacteriological swab (Letter). British Medical Journal 2, 229-230.

WICKER, R. & AVRAMEAS, S. (1969). Localisation of virus antigens by enzyme-labelled antibodies. Journal of General Virology 4, 465-471.

WILKINSON, A.E. (1951). Note on use of Stuart's transport medium for isolation of gonococcus. British Journal of Venereal Diseases 27, 200-201.

WILKINSON, H.W. & EAGON, R.G. (1971). Type-specific antigens of group B type Ic streptococci. Infection and Immunity 4, 596-604.

WILKINSON, H.W. (1972). Comparison of streptococcal R antigens. Applied Microbiology 24, 669-670.

WILKINSON, H.W. (1973). Capillary precipitin test for typing group B streptococci. Reprint instruction, Centre for Disease Control, Atlanta, Georgia, U.S.A.

WILKINSON, H.W. (1975). Immunochemistry of purified polysaccharide type antigens of group B streptococcal types Ia, Ib and Ic. Infection and Immunity 11, 845-852.

WILKINSON, H.W. (1977). CAMP-Disk test for the presumptive identification of group B streptococci. Journal of Clinical Microbiology 6, 42-45.

WILKINSON, H.W. (1978). Analysis of group B streptococcal types associated with disease in human infants and adults. Journal of Clinical Microbiology 7, 176-179.

WILSON, G.S. & MILES, A.A. (1964). Properties and nomenclature of the individual groups of haemolytic streptococci, Group B. p.713 In Topley and Wilson (Ed.), Principles of Bacteriology and immunity, vol. 1 The Williams and Wilkins Co., Baltimore.

WINTERBAUER, R.H., FORTUNE, R. & EICKHOFF, T.C. (1966). Unusual occurrence of neonatal meningitis due to group B beta-hemolytic streptococci. Pediatrics 38, 661-662.

WITTNER, M.K. & HAYASHI, J.A. (1965). Studies of streptococcal cell walls. VII. Carbohydrate composition of group B cell walls. Journal of Bacteriology 89, 398-402.